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14. ABSTRACT

The long term goal of our work is to derive kidney tissue from embryonic stem cells. This will allow us to generate patient-specific models of polycystic kidney disease, which affects many military families. Self-renewal versus differentiation decisions of nephron progenitor cells are profoundly influenced by cells in their immediate environment, the so-called progenitor cell niche. The aim of our study was to recapitulate the progenitor cell niche in vitro to provide an environment for the propagation of nephron progenitor cells derived either from embryonic mouse kidneys or human embryonic stem cells. We have used a combinatorial screening approach to identify growth factors, small molecule inhibitors and extracellular matrix components that promote proliferation of undifferentiated nephron progenitor cells derived either from embryonic tissue or from human ES cells. Using these conditions we can accomplish approximately 1:4,000 expansion, resulting in billions of cells from a starting population of only a million. Importantly, our work shows that these cells not only maintain their undifferentiated phenotype, but also retain their capacity for differentiation to nephron tubules. Thus, we can now recapitulate human nephron development *in vitro*. This represents a very significant step forward and will form the foundation for the development of polycystic kidney disease models.

15. SUBJECT TERMS
Nephron progenitor cells; stem cells; cell culture conditions

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Table of Contents

Page

1. INTRODUCTION

PKD is a common genetic disease that affects many active military personnel and their families. Treatment for PKD is currently limited to supportive therapy, dialysis and kidney transplantation. Because many PKD patients require transplantation, there is a strong incentive to increase the availability of kidney tissue. In the regenerative medicine field, two key advances have demonstrated the feasibility of a cell-based therapy approach for renal replacement: i) Functional nephrons arise from engrafted embryonic kidney cells in the adult animal, ii) ES cell treatment protocols have been developed to promote differentiation along the nephron lineage. Treatments in which autologous pluripotent stem cells are established from PKD patients and used to generate genetically corrected nephron progenitor cells for engraftment are therefore, in principle, realistic. However, a number of basic obstacles are currently holding back development of this therapeutic paradigm. An important obstacle on which this application focuses is the generation of fully functional nephron progenitor cells endowed with the capacity to form self-renewing progenitor populations following engraftment. Progress in the development of engraftable renal tissue from ES cells has been limited, largely due to shortcomings in culture protocols. In this proposal we take a novel approach to "educating" ES cell derived nephron progenitor cells using a natural cellular environment that can supply hitherto uncharacterized yet essential signals required for differentiation of fully functional nephron progenitor cells. Development of regenerative therapies is highly significant for the treatment of PKD, and for many common diseases that progress to end stage renal disease. Diabetes and cardiovascular disease are prevalent in active members of the military and their families as well as veterans, and development of regenerative therapy for kidney disease would therefore have significance far beyond PKD. This proposal tests the hypothesis that molecular cues necessary for differentiation of ES cells into fully functional nephron progenitor cells for use in cellular engraftment protocols can be provided by the progenitor cell niche.

2. KEYWORDS

Nephron progenitor cells; stem cells; cell culture conditions

3. OVERALL PROJECT SUMMARY

The objectives of the project have been fulfilled, and we now have culture conditions for both human and mouse nephron progenitor cells. We have disseminated our findings widely in the kidney development field. Several labs are now using the technology that we developed in this project and we anticipate that it already has had a significant impact on the field. A manuscript on the work has been submitted to Developmental Cell (see appendix).

SOW Objective 1: Develop a primary cell culture system for the expansion of large numbers of embryonic nephron progenitor niche cells

We used an iterative approach in which we screened additives to medium and substrates for cell growth for their effects on proliferation of nephron progenitor cells isolated from *Cited1creERT2-GFP* mice. Undifferentiated nephron progenitor cells express *Cited1* and therefore fluoresce green in this strain. This approach allowed us to easily quantify numbers of undifferentiated nephron progenitor cells. The matrices of growth factors, small molecule inhibitors and extracellular matrices yielded a single potent cocktail, which is shown in **Table 1**.

Factor	Working dose
FGF9	200ng/ml
Heparin	1ug/ml
BMP4 and 7	30ng/ml each
LDN-193189	75nM
CHIR99021	1μM
Y-27632	10μM
IGF1 and 2	20ng/ml & 2ng/ml
Matrigel	1:25 dilution
APEL medium	1X

Table 1: Composition of the NPEM medium developed for propagation of undifferentiated nephron progenitor cells in this project.

To ensure that the expanded nephron progenitor cells maintained the CITED1 and SIX2 markers of undifferentiated and also retained their capacity for differentiation to nephron tubules we performed marker analysis and differentiated organoids from consecutive cell passages (**Figure 1**).

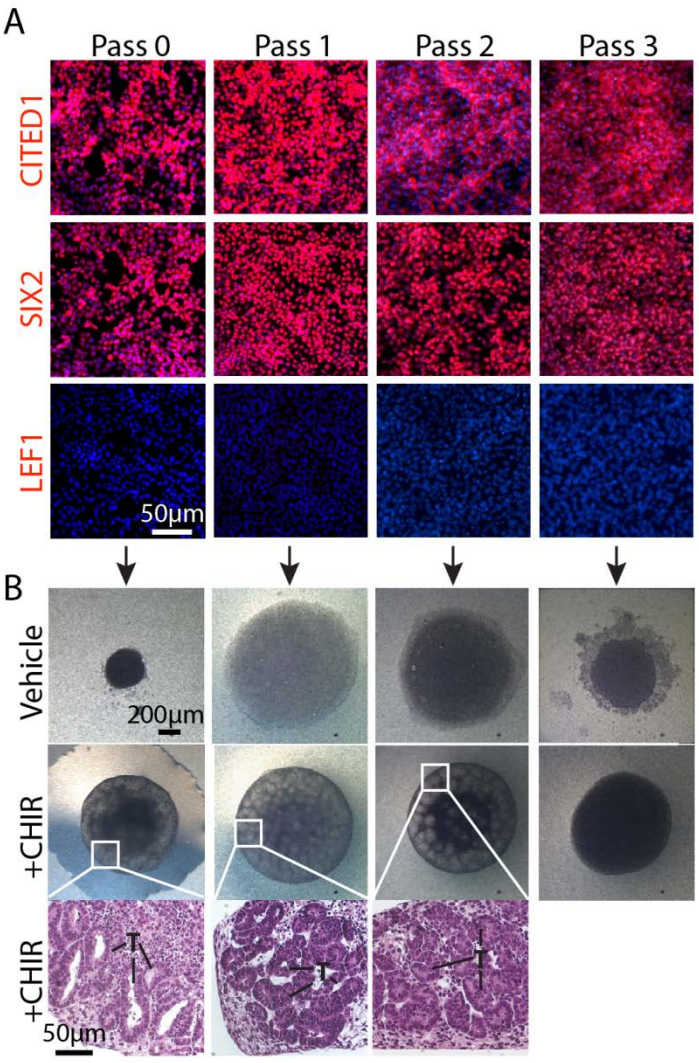

Figure 1: FGF, BMP and WNT expand functionally competent CITED1+ nephron progenitors.

(**A**) Immunostaining of CITED1+ progenitors isolated from E17.5 kidneys and expanded in NPEM through passage 3. DAPI is shown in blue.

(**B**) Stereo microscopy of aggregate cultures derived from CITED1+ progenitors cultured in monolayer in NPEM for 3 passages. Corresponding H&E staining of differentiated aggregates shows epithelial tubules (T) with lumens (bottom).

As a further confirmation of the requirements for each of the factors in NPEM medium, we systematically removed each from the medium and quantified GFP-expressing nephron progenitor cells by flow cytometry (**Figure 2**). We conclude that each factor is required.

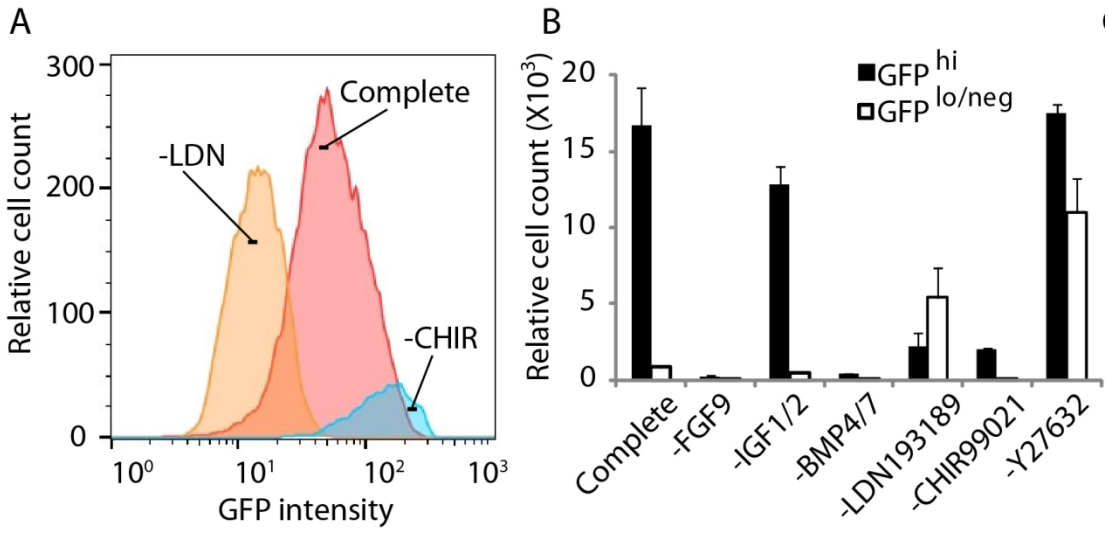

Figure 2: Culture requirements for propagation of undifferentiated nephron progenitor cells.

(A) Flow cytometry histogram of CITED1+ progenitors isolated from Cited1creERT2-EGFP reporter mice propagated in complete NPEM or in the absence of the indicated factors.

(B) Quantitation of GFP intensity (GFPHI or GFPLO/NEG) by flow cytometric analysis was used to quantify progenitor cell state in the absence of individual factors.

Using the conditions that we have developed in this project we can expand nephron progenitor cells 1:2,400 with retained potential for nephron differentiation. A typical harvest of nephron progenitor cells from one litter of mouse pups is approximately 2 million cells. From this source we can now propagate over 4 billion cells. The main limitation is currently cost, because the medium contains many expensive recombinant growth factors. In ongoing work beyond the scope of this project my laboratory is further titrating growth factor concentrations and testing cheaper replacements for the most expensive components. Although this project has been an enormous success, cost will be a significant obstacle to widespread adoption of the technology outside the small-scale research lab setting.

SOW Objective 2: Develop a primary culture system for the differentiation of pluripotent stem cells to nephron progenitors

Experiments to define a method for the differentiation of naïve stem cells to nephron progenitor cells were undertaken as described in the original proposal. Concurrent approaches utilizing co-culture of nephrogenic zone cells from mouse and a combination of small molecules and growth factors developed by a number of groups in our field were tested. Conditions developed by Melissa Little's group in Melbourne were most successful (1). Using these we were able to generate nephron progenitor cells, but these were not stable in culture. Modifying the differentiation procedure by switching cells into our NPEM medium once they had differentiated to a CITED1-expressing state stabilized their phenotype in culture and allowed us to propagate large numbers of human stem cell derived undifferentiated nephron progenitor cells (Figure 3A-C). Coculture of these cells with organs yielded some formation of tubules as described in the orginal publication. However, following propagation in our conditions, vigorous nephron

formation could be detected in organoids established by aggregating 100,000 human embryonic stem cell derived progenitors (Figure 3F-H).

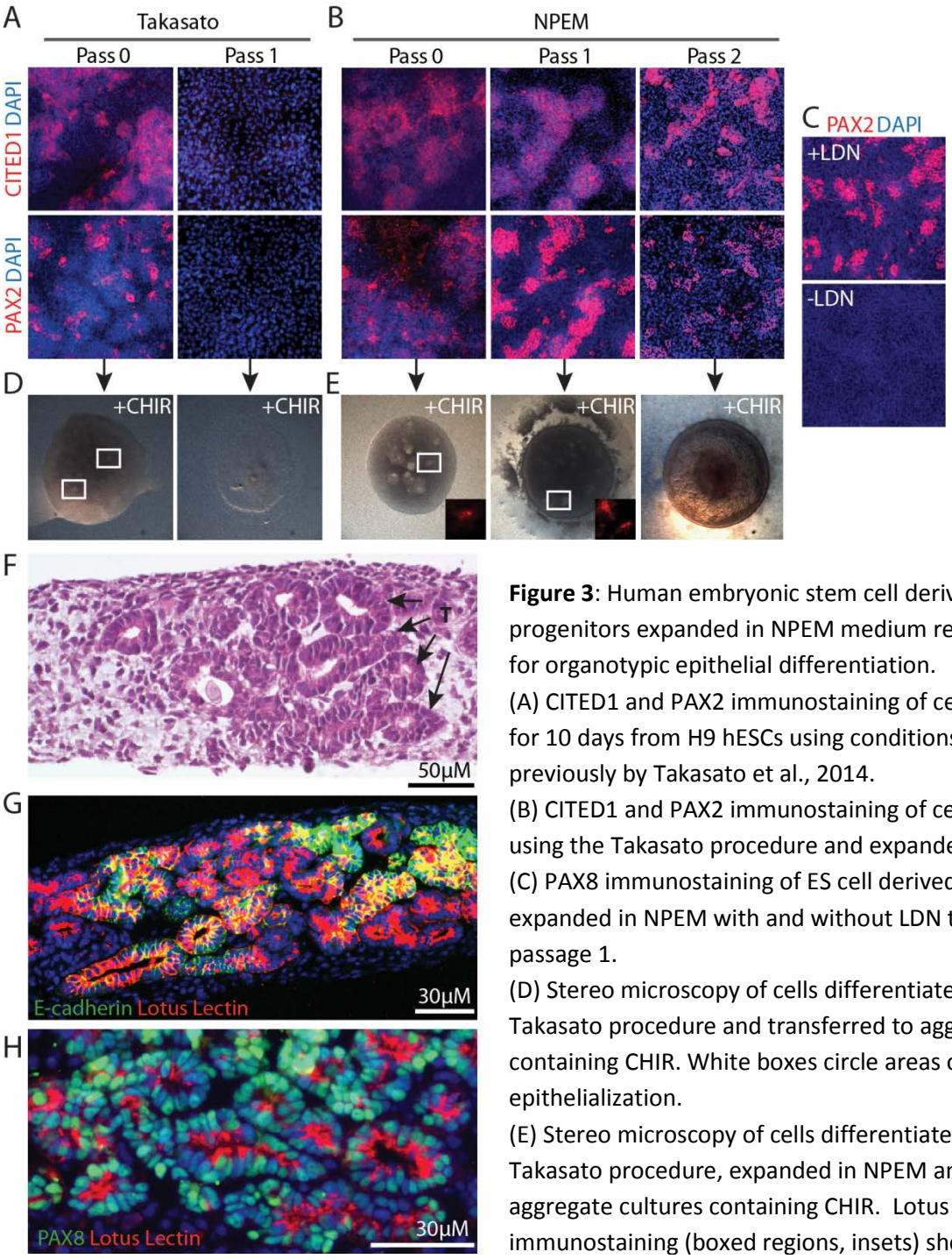

Figure 3: Human embryonic stem cell derived nephron progenitors expanded in NPEM medium retain their capacity for organotypic epithelial differentiation.

(A) CITED1 and PAX2 immunostaining of cells differentiated for 10 days from H9 hESCs using conditions reported previously by Takasato et al., 2014.

(B) CITED1 and PAX2 immunostaining of cells differentiated using the Takasato procedure and expanded in NPEM.

(C) PAX8 immunostaining of ES cell derived progenitors expanded in NPEM with and without LDN treatment at passage 1.

(D) Stereo microscopy of cells differentiated using the Takasato procedure and transferred to aggregate cultures containing CHIR. White boxes circle areas of epithelialization.

(E) Stereo microscopy of cells differentiated using the Takasato procedure, expanded in NPEM and transferred to aggregate cultures containing CHIR. Lotus lectin immunostaining (boxed regions, insets) shown in red.

(F) H&E staining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2) show extensive formation of tubules (T) with lumens (arrows).

(G) Lotus lectin and E-cadherin immunostaining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2).

(H) Lotus lectin and PAX8 immunostaining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2).

A series of engraftment experiments into SCID mice was conducted. However, the data from these in inconclusive because we have been unable to identify a cell matrix that will retain the cells *in vivo* so that they can be reliably visualized. Ongoing experiments outside the scope of this project are addressing the question of which biomaterials might be the best candidates.

4. KEY RESEARCH ACCOMPLISHMENTS

- Culture conditions for expansion of mouse nephron progenitor cells with nephron differentiation potential.
- Culture conditions for expansion of human nephron progenitor cells differentiated from H9 embryonic stem cells. These cells have the potential to differentiate into nephron tubules.

5. CONCLUSION

The results of this study are very important for the field of developmental nephrology because they represent the first successful propagation of pure undifferentiated nephron progenitor cells in culture. There are several important application for the technology that we have developed. First, it is an important step toward developing organoid-based models of polycystic kidney disease, which affects a large number of military families. Second, it is an essential step toward generating new kidney tissue for engraftment. Availability of transplant tissue does not meet demand, and military veterans are disproportionately affected by chronic kidney disease. Third, this is an enabling technology for further research into how nephron progenitor cells proliferate, which is essential for our understanding of the natural regenerative response of the kidney to injury.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

A manuscript on the work has been submitted to Developmental Cell, which is the top peer-reviewed journal in our field. Please see the appendix.

7. INVENTIONS, PATENTS AND LICENSES

We have filed an invention disclosure for the composition of NPEM.
Inventors: Leif Oxburgh and Aaron Brown
Invention title: Culture conditions for expansion of nephron progenitor cells
Application No: 61/944,982
Filing Date: 02.26.2014

8. REPORTABLE OUTCOMES

NPEM medium, which is used to propagate human and mouse nephron progenitor cells.

9. OTHER ACHIEVEMENTS

Nothing to report.

10. REFERENCES

1. Takasato M, Er PX, Becroft M, Vanslambrouck JM, Stanley EG, Elefanty AG, and Little MH. Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. *Nat Cell Biol.* 2014;16(1):118-26.

11. APPENDICES

Brown AC, Muthukrishnan SD, Oxburgh L. Functional reconstruction of the nephron progenitor niche. PDF format.

Functional reconstruction of the nephron progenitor niche

Brown AC, Muthukrishnan SD, Oxburgh L*.

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Running title

nephron progenitor niche

Summary

FGF, BMP and WNT balance embryonic nephron progenitor cell renewal and differentiation. By modulating these pathways we have created an *in vitro* niche in which nephron progenitor cells from embryonic kidneys or derived from human embryonic stem cells can be propagated. Nephron progenitor cell cultures expanded several thousand-fold in this environment can be induced to form tubules expressing nephron markers. Single cell culture reveals phenotypic variability within the early CITED1-expressing nephron progenitor cell compartment indicating that it is a mixture of cells with varying progenitor potential. Furthermore, we find that the developmental age of nephron progenitor cells does not correlate with propagation capacity, indicating that cessation of nephrogenesis is related to factors other than an intrinsic clock. This *in vitro* nephron progenitor niche will have important applications for expansion of cells for engraftment and will facilitate investigation of mechanisms that determine the balance between renewal and differentiation in these cells.

Introduction

The nephron is the filtering unit of the kidney and is essential for regulating blood urea concentration and limiting water and electrolyte loss. Nephron formation is limited to the fetal period in humans and continues to postnatal day 4 (P4) in rodents. After this period of kidney development, new nephrons can no longer be formed. Since the mature kidney lacks an identifiable population of stem cells and has a limited capacity to repair itself after injury, its long-term function relies on nephron over-capacity, which is determined during the fetal/postnatal period (Humphreys et al., 2008; Little and Bertram, 2009). Urea excretion can be augmented by dialysis, but transplantation is eventually required for patients with severe organ function impairment. End-stage renal disease affects approximately 500,000 individuals in the United States and organ availability does not match demand (Abdel-Kader et al., 2009). Technology for *ex vivo* nephrogenesis would enable therapeutic replacement of damaged kidney tissue, and provide human tissue with which to study kidney development and the origins of kidney disease. Rapid advances in reprogramming somatic cells to the pluripotent state and differentiating these through the intermediate mesoderm lineage to nephron progenitors have brought the prospect of generating patient-specific human kidney tissue within reach (Lam et al., 2013; Mae et al., 2013; Taguchi et al., 2014; Takahashi and Yamanaka, 2006; Takasato et al., 2014). While these proof-of-principle experiments have elegantly shown differentiation of nephron progenitors, the numbers of cells that they generate are relatively modest and procedures to expand these progenitors will be necessary for practical applications such as engraftment (Lam et al., 2013; Takasato et al., 2014).

The mammalian kidney develops by radial addition of new nephrons that form at the outer most cortex within a progenitor cell niche known as the nephrogenic zone. As the collecting duct branches, progenitor cell aggregates at the collecting duct tips known as cap mesenchyme are induced to differentiate into renal vesicles, polarized derivatives that are the earliest precursors of the epithelial components of the nephron (Mori et al., 2003). The continuous epithelial induction of nephron progenitor cells causes their depletion, necessitating a mechanism to balance progenitor cell renewal with epithelial differentiation, thus enabling multiple rounds of nephrogenesis. Focus on this question over the past 10

years led to the discovery of distinct cell phenotypes, or compartments, that comprise the cap mesenchyme and the specific signaling pathways on which these cells depend (Figure 1A) (Brown et al., 2013; Kobayashi et al., 2008; Mugford et al., 2009; Park et al., 2012).

The least differentiated nephron progenitor compartment is marked by the transcriptional co-activator CITED1 and transcription factor SIX2 (Boyle et al., 2008b; Self et al., 2006). Previous studies by our group and others has identified essential functions of the BMP, FGF and WNT signaling pathways in regulating the balance between renewal and differentiation in these cells (Barak et al., 2012; Blank et al., 2009; Brown et al., 2011a; Brown et al., 2013; Carroll et al., 2005; Karner et al., 2011). In this work we ask if our understanding of the signaling environment for nephron progenitor cells is sufficient to allow its reconstruction by manipulation of reported signaling pathways *ex vivo*. CITED1+/SIX2+ cells were used in a screening strategy to combinatorially test the potential of known nephrogenic zone signaling pathways to promote nephron progenitor renewal. We show that CITED1+/SIX2+ cells can be propagated in an undifferentiated state, yet retain the potential for epithelial differentiation. Thus, using a combination of recombinant proteins and small molecules we can functionally recapitulate conditions in the nephrogenic zone. Furthermore, these conditions can be extrapolated to human embryonic stem cell (hESC)-derived nephron progenitor cells, which also retain both their progenitor cell phenotype and their potential for epithelial differentiation. Hence, in addition to functionally recapitulating the signaling environment of the nephrogenic zone using a predictive approach, we have developed a method with which we can expand undifferentiated yet functionally competent human nephron progenitor cells for nephron regeneration experiments.

Results

SMAD inhibition with LDN-193189 retains nephron progenitors in the CITED1-expressing compartment. As a starting point for the development of conditions for propagation of nephron progenitor cells, we made use of a series of observations on signaling in cap mesenchyme during the terminal stage of nephrogenesis. We have previously shown that BMP7 signaling through the SMAD1/5 pathway is required for undifferentiated CITED1+/SIX2+ progenitors to transition to a CITED1-/SIX2+ state in which they are sensitized to epithelial induction by WNT/β-catenin signaling (Figure 1A) (Brown et al., 2013). In the mouse, cessation of nephrogenesis occurs shortly after birth and this is accompanied by a loss of *Cited1*+ cap mesenchyme by P2 (Figure 1B). We reasoned that SMAD1/5 signaling might increase during the terminal phase of nephrogenesis, skewing the renewal versus differentiation balance and depleting the cap mesenchyme. Immunostaining of mouse kidneys from E17.5 to P2 for activated SMAD1/5 (pSMAD1/5) showed that this is indeed the case, and that increased SMAD1/5 activation in the cap mesenchyme associates with cessation of nephrogenesis (Figure 1C).

To understand if cap mesenchyme cells in their natural signaling environment could be prevented from transitioning out of the native CITED1+ progenitor cell state, we treated newborn animals with the SMAD1/5 small molecule inhibitor LDN-193189 (LDN) during the first two postnatal days. LDN was selected over other similar analogs because it is highly specific for SMAD1/5 and has been successfully used *in vivo* (Yu et al., 2008). Immunoblot of isolated nephrogenic zone cells (NZCs) from LDN-treated animals demonstrates greater than 95% reduction in SMAD1/5 phosphorylation compared to vehicle-treated controls (Figure 1D). To measure the differentiation status of nephron progenitor cells, we used the *Cited1creERT2-EGFP* and *Six2cre-EGFP* mouse strains, which dynamically express fluorescent protein under the control of *Cited1* and *Six2* (Boyle et al., 2008b; Kobayashi et al., 2008). While untreated animals lost expression of *Cited1* and *Six2* in cap mesenchyme at P2 and P3, respectively, expression was maintained in LDN-treated pups (Figure 1E). RT-qPCR analysis of isolated nephrogenic zone cells with additional marker genes that are expressed within these two compartments supports the conclusion that the progenitor state had been rescued in LDN-treated cap mesenchymes (Figure 1F). We observed

expression of CITED1+/SIX2+ compartment-specific transcripts such as *Cited1*, *Meox1* and *Six2* and loss of markers for the CITED1-/SIX2+ and pretubular aggregate (PTA) compartments, including the WNT/β-catenin response genes *Wnt4*, *Lef1* and *Sp5*. Transcription of BMP response genes including Crossveinless-2 (*Cv2*) and several Inhibitors of differentiation (*Ids*) was also decreased, consistent with a suppression of SMAD signaling by LDN (Figure 1F). Thus, we conclude that inhibition of pSMAD1/5 activation can retain cap mesenchyme cells in a *Cited1+*/*Six2+* progenitor cell state within their natural signaling niche.

To understand if these observations can be extrapolated to CITED1+ cells in culture, we performed a series of experiments on nephron progenitor cells isolated from embryonic mouse kidneys. Using our previously developed isolation protocol, CITED1+ progenitors were harvested from embryonic mouse kidneys between the ages of E16.5 to P1 at near 100% purity, allowing interrogation of the CITED1+ population at multiple time points (Figure 2A and 2B) (Brown et al., 2013). However, CITED1 progenitor purity drops to 75% by P2, likely due to the loss of CITED1+ progenitors that occurs during cessation (Figure 2B and Figure1B). When aggregated on polycarbonate filters at the air-liquid interface and cultured in serum free medium (aggregate cultures) in the presence of the WNT/β-catenin agonists BIO (2μM) or CHIR99021 (CHIR, 3μM), these progenitors undergo robust tubulogenesis, which is dependent on endogenously produced BMP ligand (Brown et al., 2013; Osafune et al., 2006). CITED1+ progenitors isolated from P0 *Cited1creERT2-EGFP* mice underwent tubulogenesis and lost GFP expression by day 4 of CHIR treatment (Figure 2C). However, addition of LDN blocked tubulogenesis and maintained GFP expression. These results demonstrate that pSMAD1/5 inhibition can retain cultured progenitors in an undifferentiated *Cited1+* state in the presence of active BMP and WNT/β-catenin signaling, suggesting that LDN treatment will be required for *in vitro* propagation of these cells.

The developmental regulators FGF, BMP and WNT are required for maintenance, expansion and differentiation of CITED1 progenitors. To define additional factors that are required for nephron

progenitor propagation we made use of prior studies on signaling in cap mesenchyme (Table 1). We previously demonstrated that addition of either FGF1, 2, 9, or 20 promoted the maintenance and proliferation of CITED1 progenitors when cultured in monolayer on fibronectin coated wells with keratinocyte serum-free medium (Brown et al., 2011a). However, these cells lost expression of cap mesenchyme markers after 2 to 3 days and died. We chose to use FGF9 for CITED1 maintenance and proliferation as it was recently identified as a natural ligand for maintenance of nephron progenitors *in vivo* (Barak et al., 2012). Heparin was included as it facilitates the binding of FGF9 to its receptor. We have shown that BMP activation of the JNK pathway is critical for the proliferation of mouse nephron progenitors (Blank et al., 2009). Although nephron progenitors express *Bmp7,* we included recombinant BMPs to counteract the dilution of endogenous BMP7 in the culture medium. To promote protein stability in the medium we incorporated BMP4 as well as BMP7. These factors function equivalently in nephron progenitor cell renewal (Oxburgh et al., 2005). Low level WNT signaling is necessary for proliferation and renewal of CITED1 progenitors (Karner et al., 2011). Since LDN blocks cells from transitioning to the SIX2 only state in which they become sensitive to WNT-mediated epithelialization, we were able to add a low dose of CHIR (1μM) without promoting differentiation. The Rho kinase inhibitor Y-27632 was included because it increases the survival rate of dissociated stem cells during plating and passage and supports their long term maintenance (Tsutsui et al., 2011; Watanabe et al., 2007). Insulin like growth factors 1 and 2 (IGF1/2) were included because they promote cell proliferation, inhibit cell death and are important for overall kidney growth and nephron endowment in rodents (Bach and Hale, 2014; Rogers et al., 1999). To ensure compatibility with hESC differentiation approaches, we selected APEL as the basal medium and Matrigel^{TM} as the substrate because they have been used in the derivation of hESC-derived nephron progenitors (Takasato et al., 2014). When grown in this defined nephron progenitor expansion medium (NPEM), CITED1+ progenitors isolated from E17.5 mice maintained expression of CITED1/SIX2 and did not express the PTA marker LEF1 through 3 passages when split and plated at a constant cell density of 50,000 cells per cm^2 (Figure 3A). CITED1+/SIX2+/LEF1- cells proliferated vigorously at greater than 99% purity in our propagation

conditions and by passage 2 there was a 1:512 expansion of the input cell number. Thus, approximately one billion undifferentiated nephron progenitor cells can be derived from two million purified CITED1+ cells that are typically isolated from a single litter of embryonic kidneys. We conclude that our attempt to recapitulate the nephrogenic zone signaling environment for progenitor cells successfully allows extensive propagation of undifferentiated cells.

To confirm that *in vitro* propagated nephron progenitor cells retain their competence for nephron differentiation, we tested their capacity for epithelial induction under organotypic conditions in aggregate cultures containing a high concentration of CHIR (3µM). Extensive tubulogenesis was seen in cells from passages 0, 1, and 2, but cells from passage 3 did not show any tubulogenesis (Figure 3B). Aggregates of expanded progenitors cultured in differentiation conditions for 7 days revealed a molecular marker expression profile characteristic of proximal tubule differentiation (Figure 3C). This includes the sequential expression of LEF1 (PTA), ECAD (epithelialization), PAX8 (Comma and S-shaped body) and lotus lectin staining (mature proximal tubules).

During the primary seeding at a density of 50,000 cells per cm^2 (Figure 3A), we found that progenitors doubled 4 times in the first 3 days, resulting in a 16-fold expansion (Figure 3D). However, each successive passage resulted in a net loss of one doubling such that at the end of passage 2 (9 days), the bulk culture of CITED1+ progenitors had doubled 9 times leading to a 512-fold expansion. However, cells plated at a lower density (250 cells per cm^2) and cultured without passage doubled approximately 11 times over a 9 day period leading to a 2,400-fold expansion, at which time they reached confluence (Figure 3E). These cells expressed *Cited1*-driven GFP and also retained the capacity to differentiate into proximal tubules when placed into aggregate culture (Figure 3F). Thus, cell passaging of high density cultures appears to diminish both expansion and differentiation capacity suggesting that low density seeding of the primary isolate of cells yields the most functionally robust cultures. The increased doubling time of low density seeded cultures could be due to an outgrowth of progenitors with a selective advantage for expansion that is diminished in higher density culture and suggests the presence of heterogeneity in the CITED1+ compartment *in vivo*.

The majority of nephrons in the mouse kidney form after birth and nephrogenesis begins to cease when the cap mesenchyme undergoes a final wave of differentiation and becomes depleted by P4 (Hartman et al., 2007). The cap mesenchyme undergoes a distinct phase of CITED1+ depletion between P1 and P2 (Figure 1B) (Hartman et al., 2007). Because a decrease in proliferation of nephron progenitors and a continuous thinning of the cap occurs throughout nephrogenesis (Short et al., 2014), we were curious to know if CITED1+ progenitors isolated at P1 have a limited capacity for expansion caused by mechanisms underlying cessation of nephrogenesis. To test this possibility, we isolated P1 CITED1+ progenitors (Figure 2A and 2B), cultured them in NPEM, and monitored their expression by immunostaining. For each passage, P1 CITED1+ progenitors were seeded at a constant starting density (50,000 cells/cm^2) and, surprisingly, maintained robust CITED1 and SIX2 expression through 6 passages (Figure 4A). In contrast to E17.5 progenitors, CITED1+ purity dropped slightly during each expansion period, but still remained greater than 90% after 5 passages (Figure 4B). Robust differentiation was observed in aggregate cultures from each passage, even when CITED1+ purity had dropped to 65% at the end of passage 6 (Figure 4B and 4C). Initial proliferation of the fresh isolate (passage 0) was 4-fold lower for bulk P1 progenitors compared to those isolated at E17.5 (Figure 3D and Figure 4D). An interesting increase in proliferation occurred after passage 3, which subsided by passage 6. Overall, P1 progenitors doubled 12.5 times over 6 passages resulting in approximately 6,000-fold expansion, taking into account the drop in CITED1+ purity. Previous marker analysis has shown that the CITED1+ compartment contains sub-domains that display heterogeneity in gene expression (Mugford et al., 2009). *Meox1* and *Dpf3* mark a subset of CITED1+ progenitors that are physically adjacent to the differentiating CITED1-/SIX2+ population, and we therefore postulate that these markers may identify a population of CITED1+ cells poised to differentiate. Gene expression data from freshly isolated CITED1+ progenitors indicated that P1 progenitors expressed equivalent levels of *Cited1* and *Six2*, but higher levels of *Meox1* compared to E17.5, suggesting that they may have begun to shift into a more differentiated CITED1+ sub-compartment (Figure 4E). P1 progenitors also displayed a statistically significant increase in expression of the marker *Jag1*, which normally displays regionalized expression in the renal vesicle. Interestingly,

JAG1 protein cycled during passaging, suggesting that it may also play a role in undifferentiated progenitors (Figure 4A). Consistent with increased pSMAD1/5 signaling in the more medullary cap mesenchyme at P1 (Figure 1C), we observed a slight trend towards increased *Cv2* gene expression (Figure 4E). This increase in BMP-SMAD signaling would be predicted to cause increased sensitivity of CITED1+ cells to WNT-β-catenin mediated differentiation (Brown et al., 2013). However, a functional test for differentiation by measuring *Wnt4* expression in monolayer culture revealed that freshly isolated progenitors were not hypersensitive to a high concentration of BIO in the absence of additional BMP ligand, as was found for the progenitor isolate at P2, suggesting P1 CITED1+ progenitors were not yet primed for differentiation by BMP signaling (Figure 4E) and (Brown et al., 2013). Thus, P1 progenitors display a molecular marker signature consistent with increased differentiation within the CITED1+ compartment, but have greater potential to expand in bulk culture than E17.5 progenitors.

SMAD inhibition is critical to maintain nephron progenitor potential. Using flow cytometry on E17.5 CITED1+ progenitors derived from *Cited1creERT2-EGFP* mice, we evaluated the requirement for each of the culture additives to maintain cells in the undifferentiated state by subtracting them from the medium (Figure 5A and 5B). Progenitors grown in the absence of either FGF9 or BMP4/7 for 3 days failed to expand and displayed a shrunken morphology (Figure 5B and Figure S1A). Cells grown in the absence of IGF1/2 maintained CITED1 expression but grew more slowly than in the complete NPEM. In the absence of the WNT agonist CHIR, cells failed to expand but remained CITED1+ and looked morphologically similar to cells grown in complete NPEM (Figure 5B and Figure S1A). In the absence of the Rho kinase inhibitor Y-27632 there was a significant decrease in cell attachment, a corresponding increase in cell-cell contact, and importantly, a large proportion of progenitors unexpectedly transitioned out of the CITED1 compartment (Figure 5B and Figure S1A), demonstrating that inhibition of this pathway plays an important role in maintaining progenitor identity.

When LDN was subtracted from the medium, cells expanded but were largely CITED1 negative (Figure 5A and 5B). In the absence of LDN, nuclear localization of pSMAD1/5 increased dramatically, as

did transcription of the SMAD response gene *Cv2* (Figure 5C and 5D). While progenitors were still competent to undergo tubulogenesis after subtraction of IGF1/2, Y-27632 or CHIR, the absence of LDN rendered cells unable to undergo differentiation after 7 days in aggregate culture (Figure 5E and Figure S1B). In addition to losing CITED1 expression, progenitors cultured in the absence of LDN lost expression of characteristic marker genes including SIX2, PAX2, WT1 and SIX2, which may help to explain their lack of competence (Figure 5F and Figure S2). CITED1+ progenitors isolated from E13.5 kidneys also required LDN to expand while maintaining their competence to differentiate (Figure S3). These results demonstrate that the SMAD signaling branch of the BMP pathway must be quenched to maintain nephron progenitor potential.

Clonally expanded nephron progenitors are competent to form nephron tubules. We have demonstrated that bulk populations of pure CITED1+ progenitors can be expanded in NPEM while retaining their potential to undergo differentiation. We next tested a mixed culture of cells isolated from the nephrogenic zone to determine if our culture conditions confer a selective growth advantage to nephron progenitor cells. An isolation method was used that results in a mixed population of approximately 50% nephron progenitor cells, 35% cortical interstitial cells and 15% other cells with trace contamination of collecting duct cells (Blank et al., 2009; Brown et al., 2011b; Brown et al., 2013). Expansion of a bulk culture of these nephrogenic zone cells (NZCs) increased the proportion of SIX2+ cells from 50% to 85% after 2 passages (Figure 6A). Clones of CITED1+ cells expanded after 5 days in a limiting dilution assay using NZCs (Figure 6B). Twenty independent clones were expanded for 8 days and all tested positive for CITED1, SIX2 or PAX2, indicating preferential outgrowth of cells of the nephron progenitor lineage (Figure 6B). We conclude that nephron progenitors expand preferentially in NPEM over other cell types found within the nephrogenic zone and that nephron progenitor specific clones can be expanded from a single cell using our culture conditions.

High density bulk cultures of E17.5 CITED1+ progenitors were limited in their capacity to expand. Heterogeneity of gene expression suggests that the CITED1+ compartment contains cells with

varying degrees of progenitor potential (Mugford et al., 2009). To determine if the CITED1+ compartment represents a homo- or heterogeneous progenitor population, we performed a limiting dilution analysis. A total of 960 cells from a bulk population of CITED1+ progenitors purified from E17.5 kidneys, were distributed across ten 96 well plates. After attachment, the number of cells seeded per well mirrored the expected Poisson distribution (Figure S4). After 5 days, many colonies had formed and the number of cells per clone was counted to compare the doubling times of individual progenitors (Figure 6C). The growth rate observed was heterogeneous with 10% of clones doubling 7 times (17 hours per average doubling), a rate higher than that seen with our earlier low density seeding of bulk cultures (19.6 hours per average doubling). Wells containing a single colony derived from a single cell that fell within this higher proliferating category were interrogated further to determine their capacity for expansion. On day 12, 8 clones with an average cell count of 32,000 (15 doublings) were placed in aggregate culture and tested for their ability to differentiate (Figure S5A). All 8 clones formed lotus lectin positive tubules, as seen with the bulk culture studies. This demonstrates that clonally derived progenitors remain competent to differentiate after 15 doublings.

We found that many of the larger colonies started to show increased clustering of cells, which may subject individual progenitors to non-uniform culture conditions, restrict growth potential or even cause spontaneous differentiation through increased cell-cell contact. To circumvent this and to expand our clones further, we tested whether colonies could be dissociated and re-plated at a lower density in monolayer. A test clone was passed at day 11 to a new well, spread uniformly and expanded to 128,800 cells (17 doublings) after 2 more days in monolayer. When this clone was transferred to aggregate culture, it underwent robust tubulogenesis under differentiating conditions (Figure S5B), demonstrating that passage of clonally derived progenitors is possible and may extend their functional utility. In the same manner, 24 more high growth clones were split and passed to 2 wells each, expanded to confluence and either transferred to aggregate culture for differentiation or immunostained with anti-CITED1 antibody to determine purity. Figure 6D shows the total number of cells expanded from each starting progenitor and the percent of cells that were CITED1+ within each clone. Over half of the clones were greater than 90%

CITED1+ and 15 grew to more than 100,000 cells. There was a correlation between CITED1 purity and total cell expansion, with the 12 most expanded clones averaging 90% CITED1 purity and the 12 least expanding clones averaging only 60% purity. Several outlier clones (2, 7 and 17) had a lower cell number after the passage and when these cells were placed in aggregate culture, they had a reduced ability to undergo tubulogenesis compared to clones that were still expanding after the passage, suggesting that they might have become compromised (Figure 6E). Of the 24 clones, only 1 did not undergo tubulogenesis (#4), and this clone was associated with the lowest percentage of CITED1+ progenitors (4%). The remaining 23 clones underwent partial to complete tubulogenesis when subjected to differentiating conditions in aggregate culture. One healthy clone was further expanded over a period of 23 days including 2 passages and underwent extensive tubulogenesis when transferred to aggregate (Figure 6F). Confocal microscopy of this clonally derived aggregate showed numerous E-cadherin and lotus lectin positive tubules with lumens that can be visualized by optical sectioning. Overall, our results provide the first functional evidence that the CITED1+ compartment is comprised of nephron progenitor cells that display a wide variability in progenitor potential.

Expansion of functional nephron progenitor cells derived from hESCs. To understand if cellular growth in NPEM can be directly extrapolated to human cells, we repeated our analysis with nephron progenitor cells derived from hESCs using the Takasato protocol (Takasato et al., 2014). CITED1+/SIX2+/PAX2+/WT1+ cells generated using this procedure lost expression of nephron progenitor markers following a single passage (Figure 7A and Figure S6A). However, hESC-derived nephron progenitors could be passaged at least twice (1:8 split) with retained molecular marker expression using our propagation conditions (Figure 7B and Figure S6B). Subtraction of individual components from the medium revealed a critical dependence on FGF9, LDN, CHIR and Y27632 for expression of CITED1, PAX2, SIX2 and WT1 (Figure S7A). When LDN was eliminated from the medium during the passage 2 culture, cells lost expression of PAX2 (Figure 7C). When BMP4/7 was removed, PAX2 and SIX2 expression remained robust, whereas CITED1 was decreased and WT1 was

eliminated (Figure S7A). Interestingly, when both BMP4/7 and LDN were removed concurrently, PAX2 expression remained, suggesting that LDN is only necessary to retain marker expression in the presence of exogenous BMP (Figure S7B).

To evaluate the functional capacity of expanded human nephron progenitor cells, we differentiated cell aggregates from each passage. Very few tubules formed from cells expanded during passages 0 and 1 in either medium, although the tubules that did form stained positive for lotus lectin (Figure 7E). In contrast, cells cultured through passage 2 in NPEM underwent robust differentiation forming many tubules containing lumens (Figure 7E and 7F). We found staining for both E-cadherin and lotus lectin in differentiated tubules and observed that tubules frequently expressed both of these markers (Figure 7G). When found in tubules without E-cadherin, lotus lectin staining was confined to the luminal side of tubules, where it normally stains L-fucose present on the surface of the microvilli that form the brush border. This suggests that these tubules have increased surface area, which is necessary for the resorptive and flow sensing functions of proximal tubules *in vivo*. We also observed persistent nuclear expression of PAX8, which is normally expressed in proximal and distal convoluted tubules and loops of Henle in humans, but is decreased or absent in adult mouse kidneys (Figure 7H) and (Tong et al., 2009). PAX8 staining also shows alternating expression intensity in neighboring cells within a tubular structure, similar to that observed in human proximal tubules (Tong et al., 2009). In tubules co-expressing both markers, lotus lectin often overlapped with E-cadherin staining at cell -cell junctions, but displayed stronger expression towards the luminal side (Figure 7G). Since this pattern was only found in E-cadherin positive tubules, and E-cadherin expression is normally decreased prior to terminal differentiation of the proximal tubule in mice and rats (Prozialeck et al., 2004), these structures may represent immature tubules where microvilli have not yet formed and in which expression of these two markers has not yet segregated. Another possibility is that the immediate precursors to proximal and distal tubule epithelial cells (LTL-/ECAD+) have become intermixed and given rise to a number of hybrid tubules within the organoid culture, rather than becoming regionally restricted to a single tubule type. Overall, we have demonstrated that NPEM expands ES cell derived human nephron progenitors that are capable of robust

epithelialization and formation of tubules with lumens that display expression of markers normally associated with human proximal tubules.

Discussion

We have shown that the nephrogenic zone cell signaling environment can be recreated *in vitro* for extensive propagation of undifferentiated nephron progenitor cells. Modulation of FGF, BMP, WNT and ROCK signaling pathways was necessary to maintain cells in the CITED1+ state with epithelial differentiation potential. To our knowledge, this is the first completely defined culture system for the expansion of functionally competent nephron progenitors. Importantly, this system can be used for the expansion of embryonic stem cell-derived human nephron progenitor cells. Cell signaling requirements for mouse and human nephron progenitor cells are similar in all aspects except the requirement for BMP. Subtraction of BMP from the culture medium of human cells resulted in only a partial loss of the progenitor marker profile compared to the complete loss observed for mouse progenitors. Published differentiation protocols for human embryonic stem cells generate at most 50% nephron progenitor cells, and approximately half of the culture remains undefined (Lam et al., 2013; Mae et al., 2013; Takasato et al., 2014). It therefore seems likely that cells in the culture may be producing BMPs and other factors that mask the effects of BMP7 withdrawal. Development of purification procedures for human nephron progenitor cells will be necessary to directly compare the signaling requirements of derived human nephron progenitor cells with CITED1+ cap mesenchyme cells.

BMP signaling in nephron progenitor cell maintenance

FGF, BMP and WNT each influence renewal and differentiation of the nephron progenitor cell, but how the cell interprets these signals depends on its differentiation state as well as concurrent signaling from the surrounding niche (Brown et al., 2013; Das et al., 2013; Fetting et al., 2014; Karner et al., 2011). BMP7 promotes nephron progenitor proliferation through a MAPK pathway, whereas pSMAD signaling transitions progenitors out of the CITED1+ compartment (Blank et al., 2009; Brown et al., 2013). Molecular mechanisms that determine the balance of MAPK versus pSMAD activation by BMP7 in nephron progenitors are not understood. However, recent data suggests that FGF signaling through PI3K/MAPK may repress pSMAD1/5 signaling in unprimed cap mesenchyme (Motamedi et al., 2014). In

our cultures, pSMAD1/5 persists in the presence of exogenously added FGF9, suggesting that additional niche factors present in the developing organ are required for FGF-mediated suppression of pSMAD in the CITED1+ compartment. A key factor in the development of our culture procedure was the addition of the small molecule LDN which blocks pSMAD activity and prevents progenitors from exiting the CITED1+ compartment, while still allowing proliferation and survival signals provided by BMP stimulation.

Functional heterogeneity within the CITED1+ progenitor population

Gene expression profiling of the CITED1+ cap mesenchyme suggests that this is not a homogenous cell population (Mugford et al., 2009). *Meox1* and *Dpf3* are expressed in a specific subpopulation of CITED1+ progenitors adjacent to the more differentiated CITED1-/SIX2+ compartment and are not expressed in CITED1+ progenitors in more cortical cap mesenchyme. While the functions of these transcription factors during kidney development are unknown, their localized expression indicates that the CITED1+ population may be phenotypically heterogeneous, perhaps with one renewing sub-compartment, and one sub-compartment in the process of exiting the CITED1+ state. Our analysis of single cells cultured for 5 days revealed a largely binomial distribution of cellular doubling, supporting functional heterogeneity of the CITED1+ cap mesenchyme population. Clones derived from the most rapidly dividing group could be expanded to several hundred thousand cells that retained the potential for epithelial differentiation. It seems probable that these highly proliferative clones derive from cells within the CITED1+ compartment with extensive progenitor potential that might function as "super progenitors" from which the bulk of CITED1+ cap mesenchyme cells are derived. Lineage analysis of the cap mesenchyme using a tamoxifen-inducible *Cited1-creERT2*;*Rosa26R* strain shows that a high proportion of cells labeled at E13.5 are retained in the cap mesenchyme at E19.5, suggesting the presence of a self-renewing sub-population (Boyle et al., 2008a). An alternate possibility is that our medium formulation provides an advantage to more differentiated cells within the CITED1+ compartment. In support of this, CITED1+ progenitors derived from P1 kidneys could be expanded considerably further than those

derived at E17.5, while having a lower proliferative index and higher levels of *Meox1*. Single cell transcriptome analysis coupled with phenotyping of a large number of cap mesenchyme cells will be required to discern the biological basis for the heterogeneity that we see within the CITED1+ population.

The nephrogenic niche regulates nephron progenitor lifespan

Recent high resolution studies indicate that cap mesenchyme displays progressively decreased proliferation and thinning throughout development until it is depleted (Short et al., 2014). Based upon this model and the average cell cycle lengths calculated for the cap mesenchyme (33 hours at E17.25), E17.5 progenitors in the CITED1+ compartment would on average be expected to expand no more than 2 doublings prior to cessation. However, in isolation, differentiation-competent CITED1+ progenitors were able to divide in bulk 9 times. Those that had undergone limiting dilution and selection could double even further, up to 17 times. Furthermore, CITED1+ progenitors isolated at P1, while possessing a lower proliferative index as expected, underwent more than 12 doublings in bulk culture. If progenitor renewal was internally regulated by a predetermined biological clock that counts the number of divisions, we would expect cells isolated from E17.5 to double more than those isolated at P1, but this is not the case. Instead, we speculate that the reduced rate of proliferation in the P1 cells may underlie their depletion, perhaps because epithelial induction by collecting duct tips is not reduced proportionally, leading to cap mesenchyme exhaustion. Alternately, our findings could suggest that the niche rather than an internal clock determines the number of doublings a CITED1+ cell may undertake. Removing the cells from this niche would relieve this signal resulting in similar proliferative capacity for cells isolated at different developmental stages.

Conclusions

This work demonstrates that our understanding of the nephrogenic zone signaling environment is now sufficiently advanced to allow us to emulate the niche *in vitro*, providing a means for controlled expansion of nephron progenitor cells. These cells can now be produced in large quantities facilitating

studies of nephron formation in a variety of biological matrices with the ultimate goal of tissue engraftment

Experimental procedures

Cell culture. CITED1+ progenitors were purified from NZCs derived from E17.5 kidneys as previously described (Brown et al., 2011b; Brown et al., 2013). CITED1+ progenitors were cultured in monolayer on hESC qualified Matrigel^{TM} coated plates (Corning). For human or mouse passage, cells were dissociated by incubation with TrypLE (Life Technologies) for 2 minutes at 37C, washed and spun 2X at 300g in autoMACs running buffer (Miltenyi) prior to resuspension in NPEM as described in Table 1. NPEM is changed every 2 days. Cells were differentiated in aggregate culture with CHIR (3μM) in medium as previously described (Brown et al., 2013).

Immunofluorescence and microscopy. Tissue sections, monolayer cultures and aggregates were immunostained as previously described (Blank et al., 2009; Brown et al., 2013). Antibodies were used at 1:100 dilution and include CITED1 (NeoMarkers); pSMAD1/5 (Cell Signaling Technology); SIX2 (Proteintech); LEF1 (Cell Signaling Technology); PAX2 (Proteintech); PAX8 (Proteintech); E-cadherin (BD Transduction Laboratories) and lotus lectin staining at 1:200 (Vector Laboratories). Live images of GFP+ progenitors from *Cited1creERT2-EGFP* mice in monolayer or aggregate culture were imaged with epi-fluorescent and fluorescent stereo microscopes, respectively.

Quantitative PCR. RNA purification, cDNA synthesis, and quantitative PCR were performed as previously described (Brown et al., 2011a). All raw data are normalized to β-actin expression, and fold changes are relative to the vehicle control.

Flow cytometry. CITED1+ progenitors were purified from GFP+ kidneys isolated from *Cited1creERT2-EGFP* x ICR mice and cultured as described in the text. GFP fluorescence intensity and cell counts were collected on a FACSCalibur (BD) and data were analyzed using FlowJo software.

Statistical methods

For qPCR, P-values shown were calculated using a two-tailed heteroscedastic Student's t-test and $P<0.05$ was considered significant. Error bars represent standard deviation for technical replicates derived from NZCs of 20-24 pooled kidneys or standard error for biological replicates derived from 3 independent mouse litters of pooled kidneys. For flow cytometry and cell count experiments, error bars represent average values ±SD calculated from three culture well replicates. CITED1+ purity was determined from at least 3 independent images by normalizing to the number of DAPI-stained nuclei in each field using ImageJ with error bars representing the mean ±SD.

Mouse strains and treatments. Animal care was in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals and protocols were approved by the Institutional Animal Care and Use Committee of Maine Medical Center. CITED1 progenitors were derived from kidneys of *Cited1creERT2-EGFP* x ICR (Institute for Cancer Research mice) heterozygous mice. *Cited1creERT2-EGFP* and *Six2cre-EGFP* mouse strains are maintained on an FVB/NJ background (Boyle et al., 2008b; Kobayashi et al., 2008). Pregnant mice were injected at 12 hour intervals at the times indicated with 3mg/kg LDN-193189 in 20ul of DMSO/PBS.

Author contributions

A.C.B. and L.O. designed research; A.C.B. and S.D.M. performed research; A.C.B., S.D.M. and L.O. analyzed data; and A.C.B. and L.O. wrote the paper.

Supplemental Figures

Supplemental information includes 7 figures.

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Figure legends

Figure 1| SMAD inhibition with LDN-193189 retains nephron progenitors in the CITED1 compartment *in vivo*.

(A) Schematic of cap mesenchyme compartments and key signaling pathways required for their maintenance and differentiation.

(B) *Cited1creERT2-EGFP* kidneys harvested at postnatal stages. Fluorescent imaging of GFP expression in cap mesenchymes "C" shown.

(C) Immunostaining of pSMAD1/5 (arrows) in kidney sections isolated from E17.5 to P1 (arrows). Cap mesenchymes are outlined with dotted red lines.

(D) pSMAD1/5 immunoblot of NZCs after intraperitoneal injection of P0 pups with either vehicle or 3mg/kg of LDN twice daily until P2. Percent remaining after LDN treatment quantified in graph. NZCs were isolated from 4 kidney pairs per treatment group and pooled.

(E) Fluorescent imaging of kidneys from *Cited1* or *Six2* EGFP reporter mice in vehicle and LDN treated animals. Representative image from 4 kidney pairs per group shown.

(F) Cap mesenchyme marker analysis of isolated nephrogenic zone cells by qPCR. Data represent the mean ±SD of qPCR technical replicates from 5 (DMSO) and 6(LDN) pooled kidney pairs.

Figure 2| SMAD inhibition with LDN-193189 retains nephron progenitors in the CITED1 compartment *in vitro*.

(A) CITED1 immunostaining of freshly plated monolayer cultures of purified progenitors isolated at developmental time points.

(B) Quantitation of CITED1+ progenitor purity from images shown in (A).

(C) Stereo microscopy and GFP expression of purified CITED1+ progenitors in aggregate culture isolated from *Cited1creERT2-EGFP* kidneys and treated with CHIR (3μM) and LDN (75nM).

Figure 3| FGF, BMP and WNT expand functionally competent CITED1+ nephron progenitors.

(A) Immunostaining of CITED1+ progenitors isolated from E17.5 kidneys and expanded in NPEM through passage 3. DAPI is shown in blue.

(B) Stereo microscopy of aggregate cultures derived from CITED1+ progenitors cultured in monolayer in NPEM for 3 passages. Corresponding H&E staining of differentiated aggregates shows epithelial tubules (T) with lumens (bottom).

(C) Time course images of expanded CITED1+ progenitors differentiated in aggregate culture.

(D) Number of CITED1+ progenitors present after each 3 day culture period with 50,000 cells per cm^2 seeding density.

(E) Number of CITED1+ progenitors present at the end of passage 0 (9 days) when plated at a low seeding density (250 cells/cm^2).

(F) GFP expression and lotus lectin (LTL) staining of CITED1+ progenitors expanded 2400 fold (from experiment in (E)) and transferred to aggregate culture.

Figure 4| Slow cycling late nephron progenitors expand and retain differentiation potential.

(A) Immunostaining of CITED1+ progenitors isolated from P1 kidneys and cultured in NPEM through passage 6. DAPI is shown in blue.

(B) Corresponding aggregate cultures derived from cell passages shown in (A).

(C) Percent of CITED1+ cells remaining after each passage.

(D) Number of CITED1+ progenitors present after each 3 day culture period with 50,000 cells per cm^2 seeding density.

(E) Expression of cap mesenchyme transcripts of freshly isolated CITED1+ progenitors.

(F) *Wnt4* expression in CITED1+ progenitors starved in keratinocyte basal medium and treated with BIO (0.5μM) for 6 hours. Average values ± SEM in (E) and (F) shown.

Figure 5|SMAD inhibition maintains nephron progenitor potential.

(A) Flow cytometry histogram of CITED1+ progenitors isolated from *Cited1creERT2-EGFP* reporter mice propagated in complete NPEM or in the absence of the indicated factors.

(B) Quantitation of GFP intensity (GFP^{HI} or $GFP^{LO/NEG}$) by flow cytometric analysis was used to quantify progenitor cell state in the absence of individual factors.

(C) pSMAD1/5 immunostaining of CITED1+ progenitors grown in NPEM with and without LDN or BMP.

(D) *Cv2* expression in CITED1+ progenitors grown in NPEM in the presence (complete) and absence of LDN over 72 hours. Mean ±SD shown.

(E) Aggregate culture of CITED1+ progenitors isolated from *Cited1creERT2-EGFP* reporter mice that were initially expanded for 3 days in monolayer in the presence or absence of LDN.

(F) Immunostaining of CITED1+ progenitors expanded in NPEM for 3 days in the presence or absence of LDN.

Figure 6| NPEM supports clonal expansion of functional nephron progenitors from a heterogeneous CITED1+ pool.

(A) SIX2 immunostaining of isolated NZCs (pass 0 and 2) and CITED1+ progenitors (pass 2) expanded in NPEM.

(B) Stereo microscopy and immunostaining of single cell derived colonies obtained from NZCs seeded in NPEM.

(C) Graphical representation of cell doublings in colonies seeded by single CITED1+ progenitor.

(D) Number of cells recovered (black bars, left y-axis) and percent CITED1+ (white boxes, right y-axis) of single cell seeded colonies after passage 1.

(E) Phase contrast (left) and lotus lectin immunostain (right) of 24 clones (from (D)) differentiated with CHIR (3μM) in aggregate culture.

(F) Stereo (top) and confocal (bottom) microscopy of an aggregate derived from a single CITED1+ progenitor after propagation in NPEM for 23 days through 2 passages (LTL – lotus lectin).

Figure 7| Human embryonic stem cell derived nephron progenitors expanded in NPEM medium retain their capacity for organotypic epithelial differentiation.

(A) CITED1 and PAX2 immunostaining of cells differentiated for 10 days from H9 hESCs using conditions reported previously by Takasato et al., 2014.

(B) CITED1 and PAX2 immunostaining of cells differentiated using the Takasato procedure and expanded in NPEM.

(C) PAX8 immunostaining of ES cell derived progenitors expanded in NPEM with and without LDN treatment at passage 1.

(D) Stereo microscopy of cells differentiated using the Takasato procedure and transferred to aggregate cultures containing CHIR. White boxes circle areas of epithelialization.

(E) Stereo microscopy of cells differentiated using the Takasato procedure, expanded in NPEM and transferred to aggregate cultures containing CHIR. Lotus lectin immunostaining (boxed regions, insets) shown in red.

(F) H&E staining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2) show extensive formation of tubules (T) with lumens (arrows).

(G) Lotus lectin and E-cadherin immunostaining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2).

(H) Lotus lectin and PAX8 immunostaining of CHIR treated aggregate cultures containing cells expanded in NPEM (pass 2).

Table 1: Summary of factors used in nephron progenitor expansion medium (NPEM)

Factor	Working dose	Function	References
FGF9	200ng/ml	Renewal and proliferation	(Barak et al., 2012; Brown et al., 2011a)
Heparin	1ug/ml	FGF signaling activity	(Venkataraman et al., 1996)
BMP4 and 7	30ng/ml each	proliferation	(Blank et al., 2009)
LDN-193189	75nM	SMAD inhibitor of differentiation	(Yu et al., 2008)
CHIR99021	1μM	WNT agonist, renewal and proliferation	(Karner et al., 2011)
Y-27632	10μM	ROCK inhibitor, survival during plating	(Tsutsui et al., 2011; Watanabe et al., 2007)
IGF1 and 2	20ng/ml & 2ng/ml	Cell growth and proliferation	(Bach and Hale, 2014; Rogers et al., 1999)
Matrigel	1:25 dilution	ECM, cell attachment	(Takasato et al., 2014)
APEL	1X	Basal stem cell medium	(Takasato et al., 2014)

Figure 1
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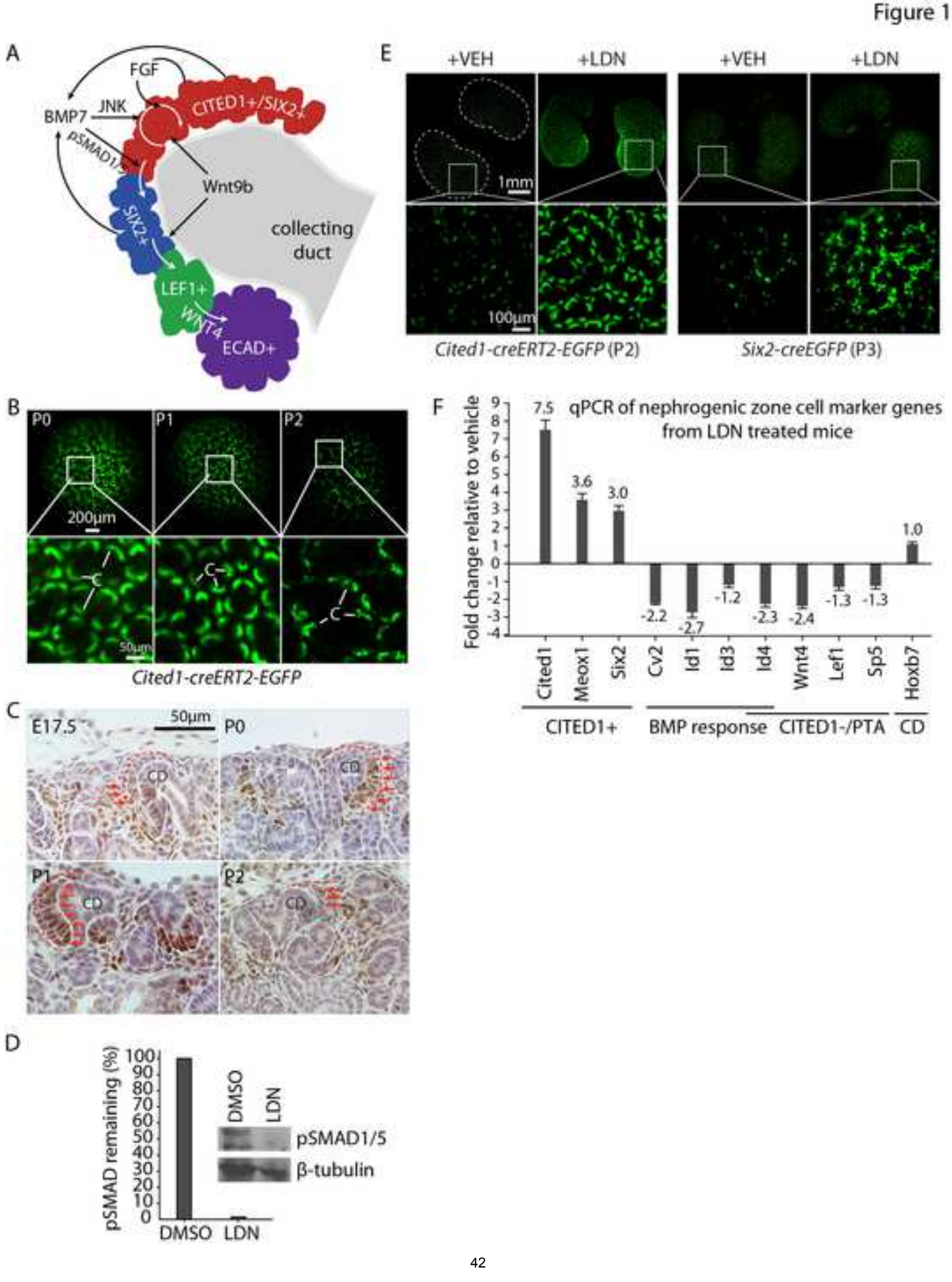

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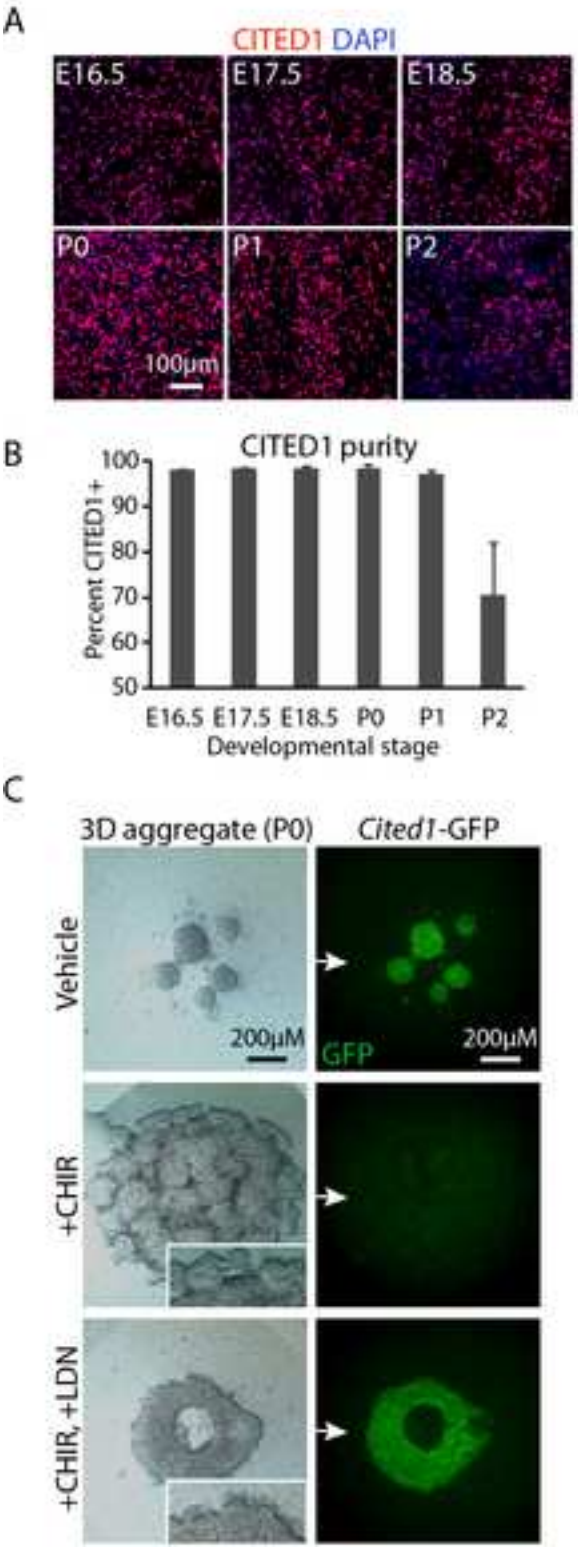
Figure 2



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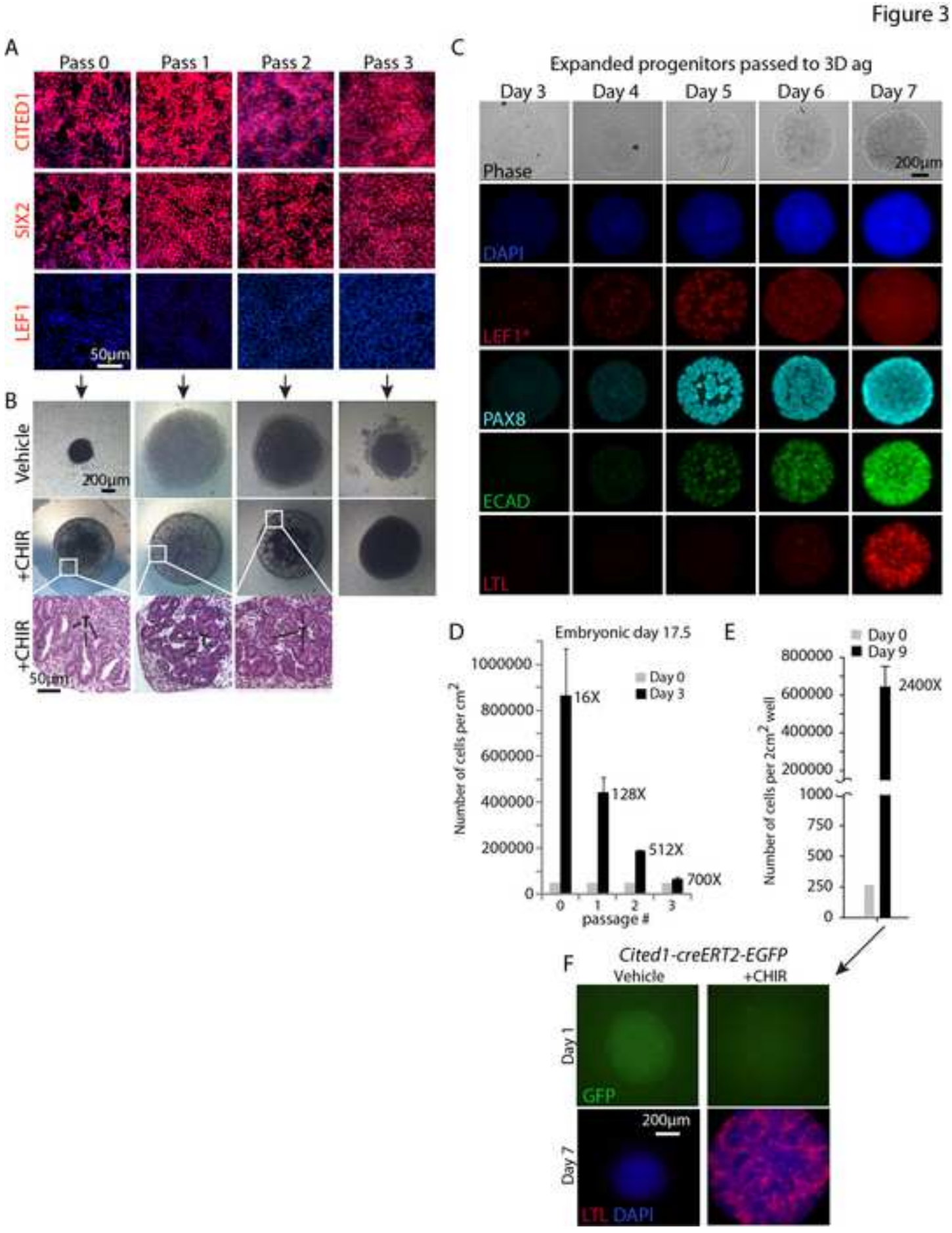

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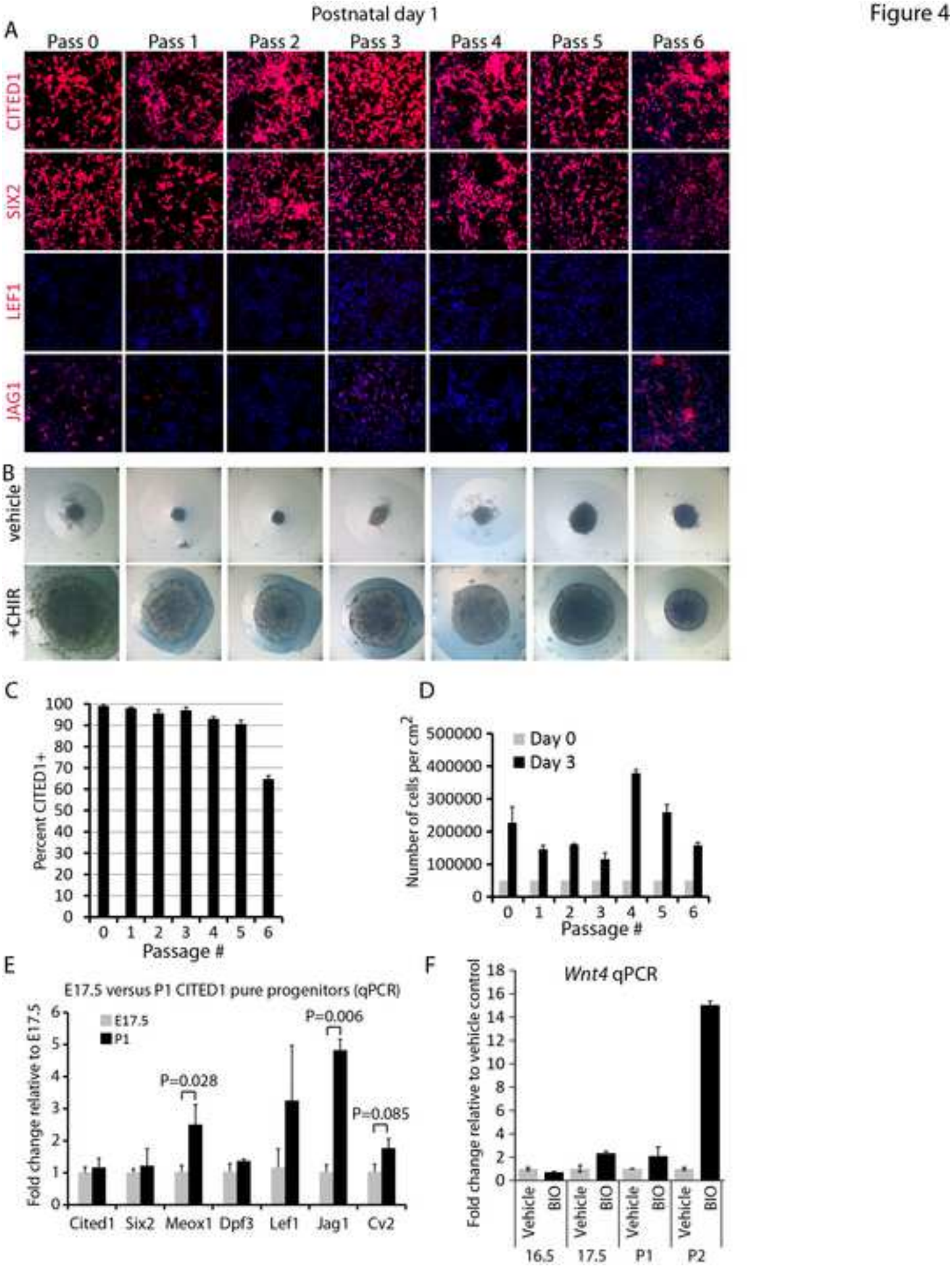

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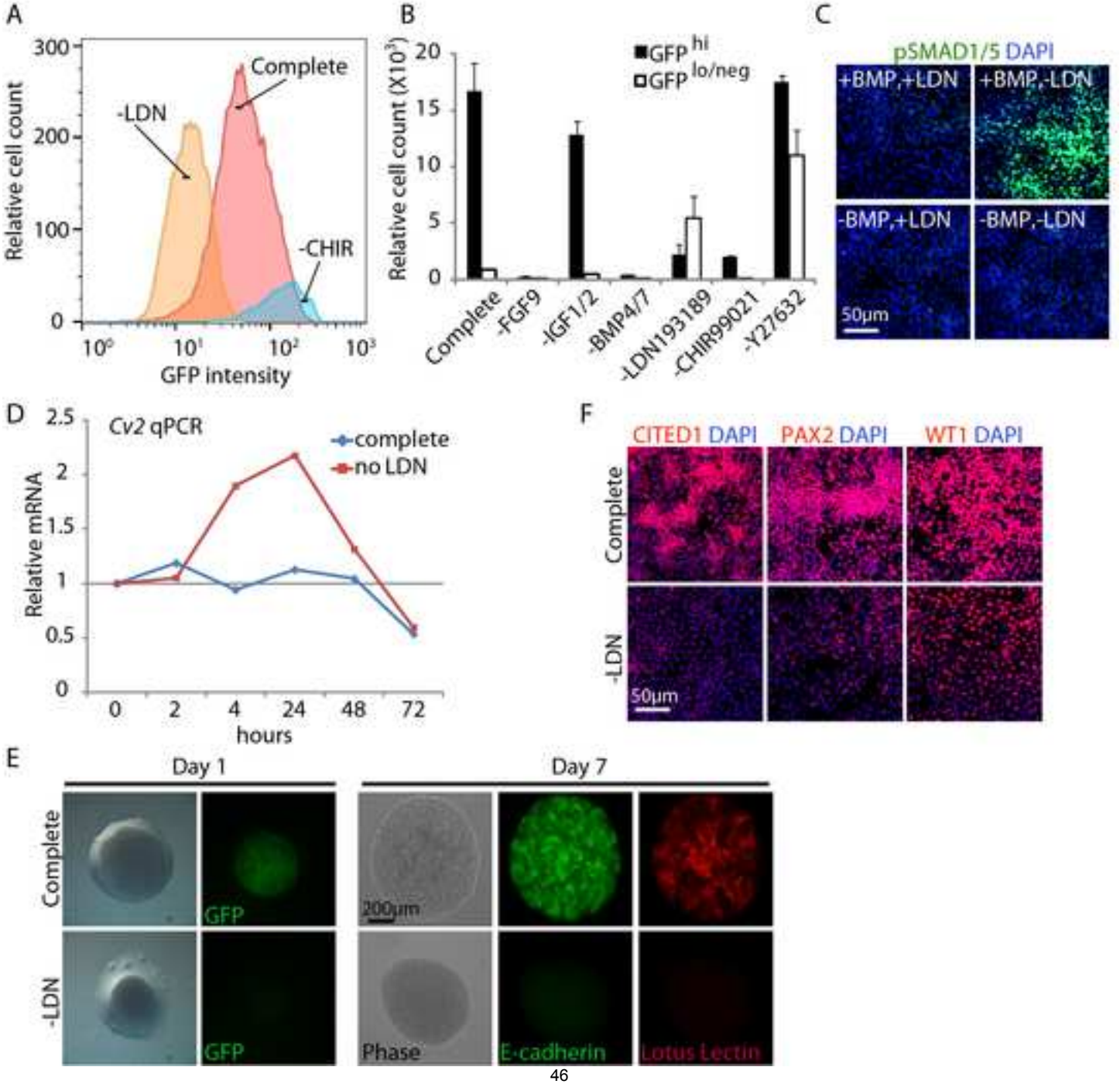

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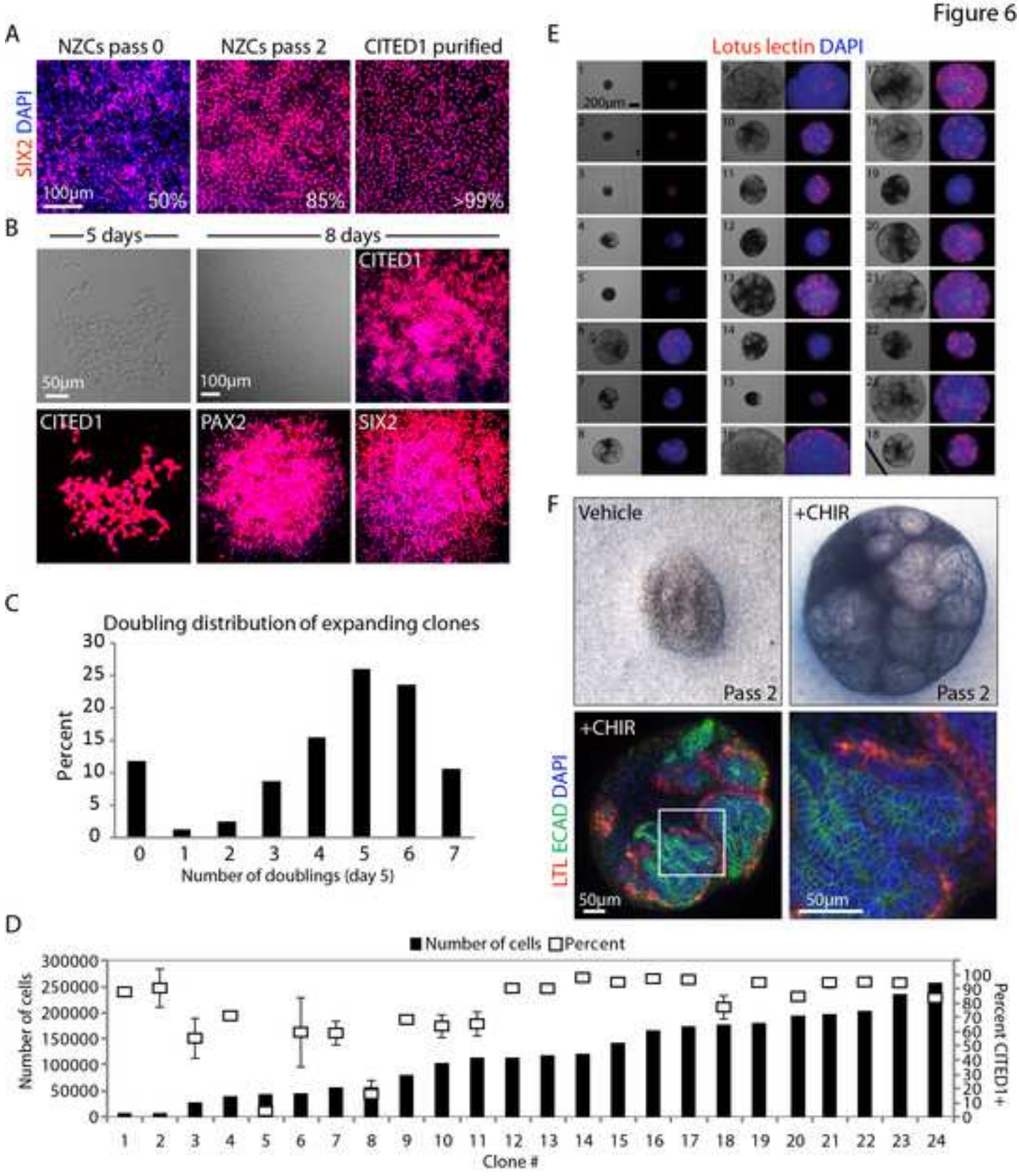

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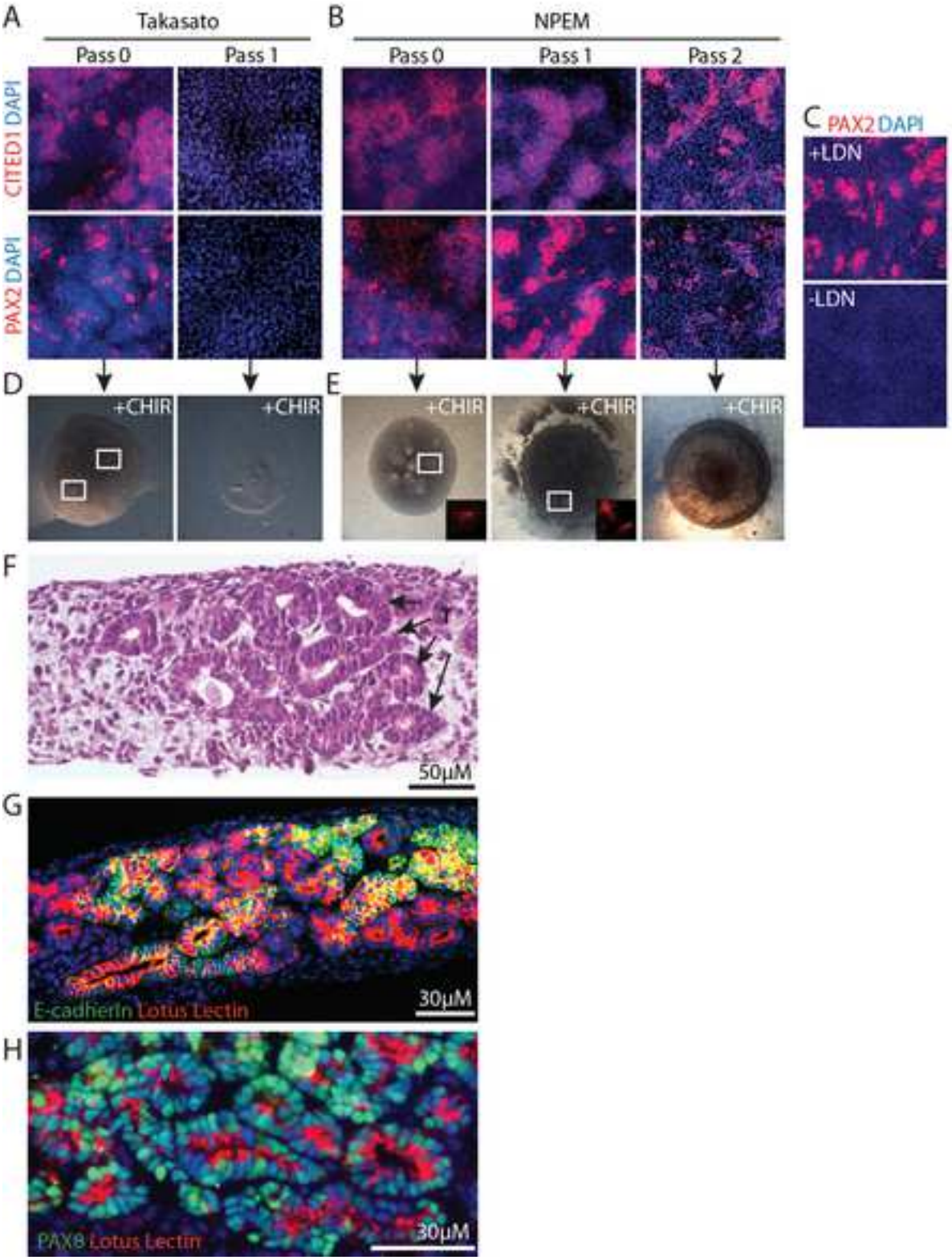


Figure S1

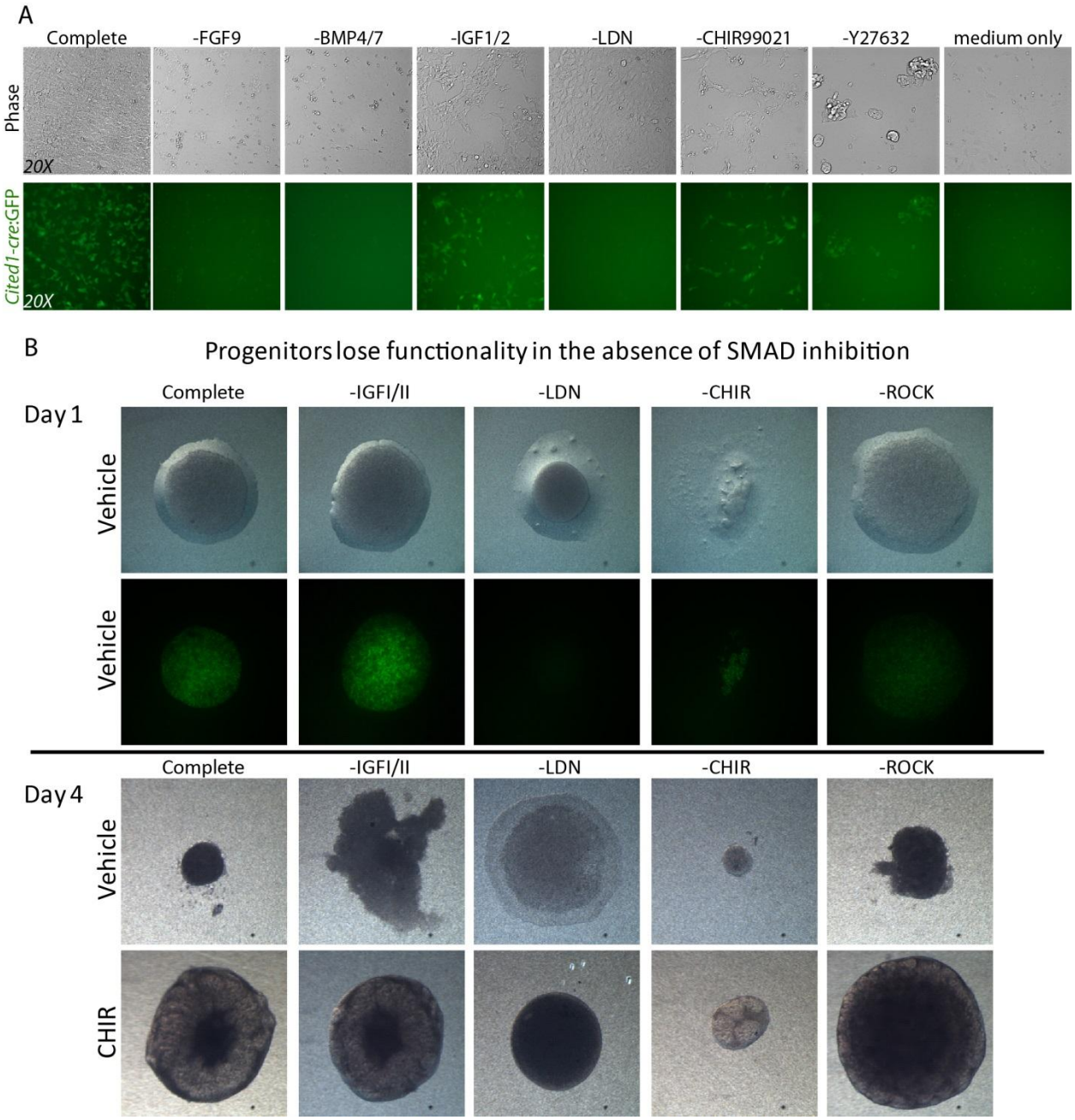


Figure S1| Nephron progenitor cultures growth in the absence of individual factors.
(A) Individual factors were removed from NPEM and purified CITED1 progenitors from *Cited1creERT2-EGFP* x ICR mice were cultured in monolayer for 3 days. Cultures contain a 50/50 starting mix of GFP+ and GFP- cells. Top panels: Phase microscopy of cell morphology after 3 days in the absence of individual factors. Bottom panels: Corresponding immunofluoresence of GFP signal after 3 days in culture.
(B) GFP immunofluorescence and corresponding light microscopy of 3D aggregates grown for 1 and 4 days respectively. Individual factors were removed from NPEM and purified CITED1 progenitors from *Cited1creERT2-EGFP* x ICR mice were cultured for 3 days in monolayer culture. Cells were dissociated and spotted in 3D aggregate on floating filters for the indicated times. Top panels: Vehicle only control

shown after 1 day in culture. Bottom panels: Tubulogenesis can be seen when cultures are treated with CHIR, except when monolayer cultures were grown in the absence LDN. 3 monolayer replicates were pooled per 3D aggregate. 1 of 2 experimental replicates shown. Note: FGF9 and BMP4/7 minus cultures died during the monolayer phase.

Figure S2

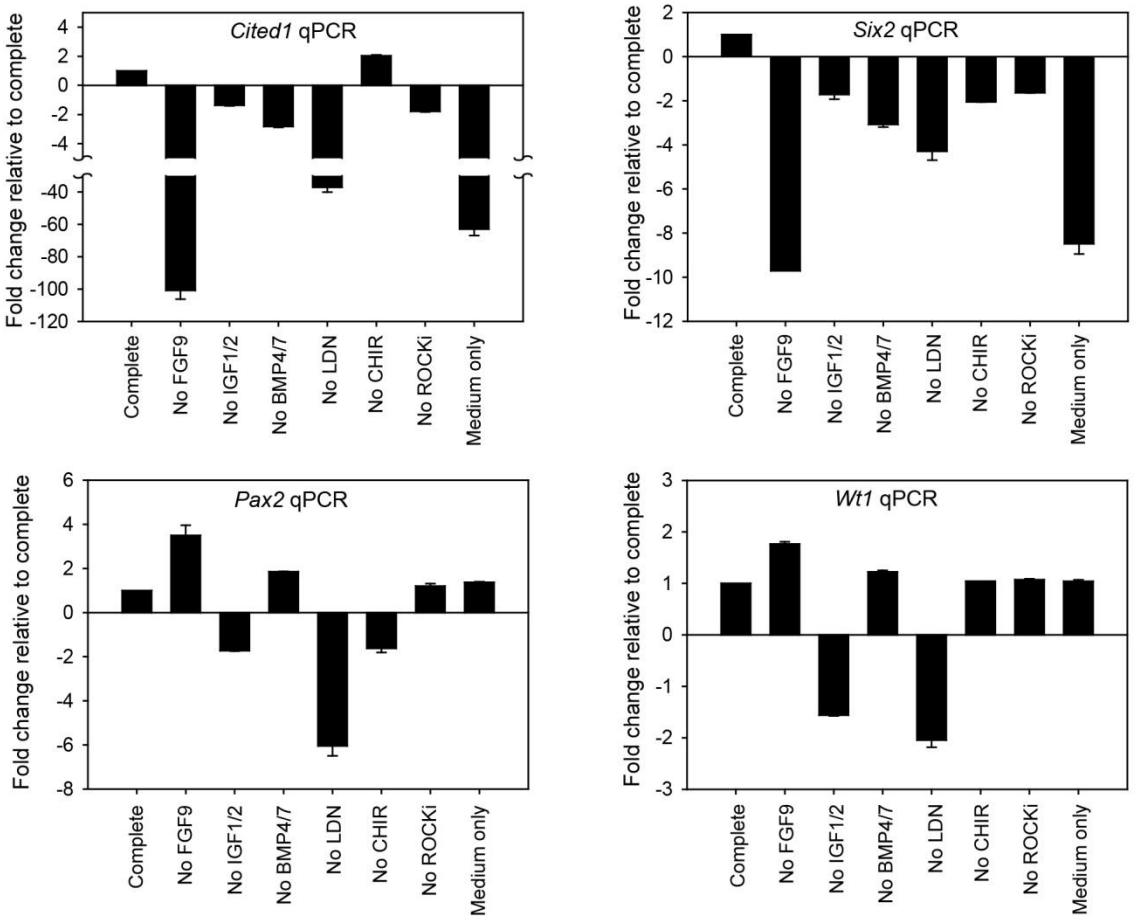


Figure S2| Nephron progenitor marker expression in cultures grown in the absence of individual factors. Purified CITED1 progenitors were grown in NPEM in the absence of individual factors for 3 days in monolayer culture. Cells were lysed and gene expression was measured by quantitative PCR. Fold changes are relative to cells grown in complete NPEM for each primer set. Results shown represent 3 pooled culture replicates derived from 20-24 pooled embryonic kidneys.

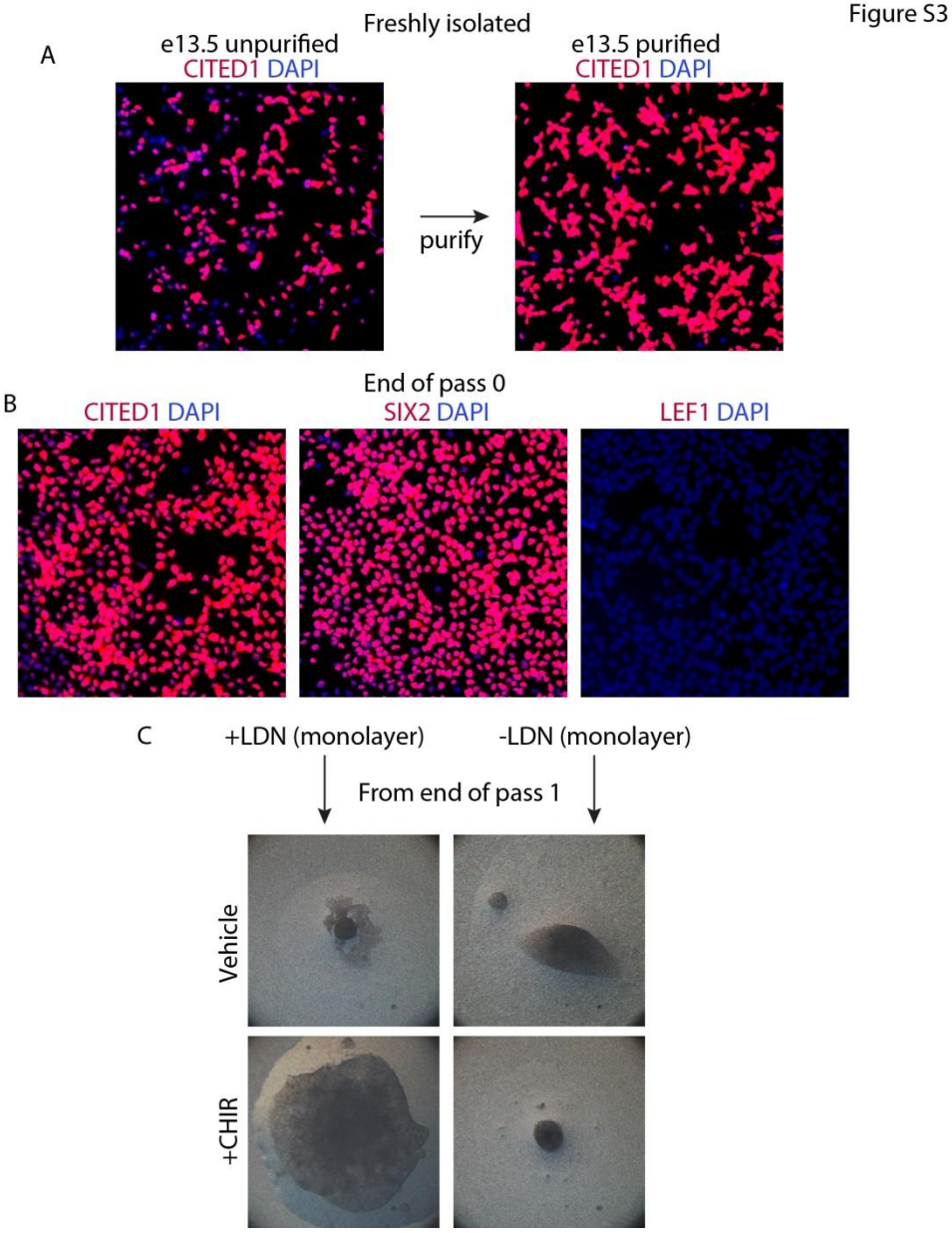


Figure S3| LDN is required to maintain CITED1+ progenitors derived from E13.5 embryonic kidneys.
(A) Progenitors purified from nephrogenic zone cells isolated from E13.5 kidneys show CITED1+ enrichment.
(B) Purified CITED1 progenitors expanded in NPEM for 3 days retain expression of CITED1, SIX2, but not LEF1 protein.
(C) Purified CITED1 progenitors expanded in monolayer cultures with NPEM containing LDN undergo differentiation when transferred to 3D aggregate culture for 7 days after treatment with a high dose of CHIR (left panels). CITED1 progenitors expanded in monolayer cultures with NPEM, but without LDN lose their potential to differentiate (right panels).

Figure S4

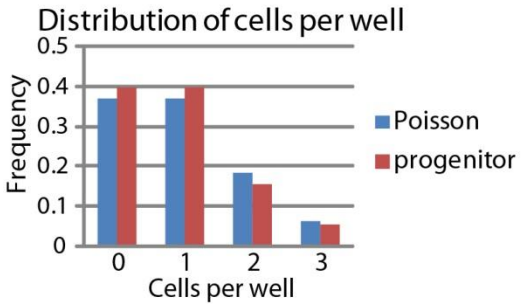
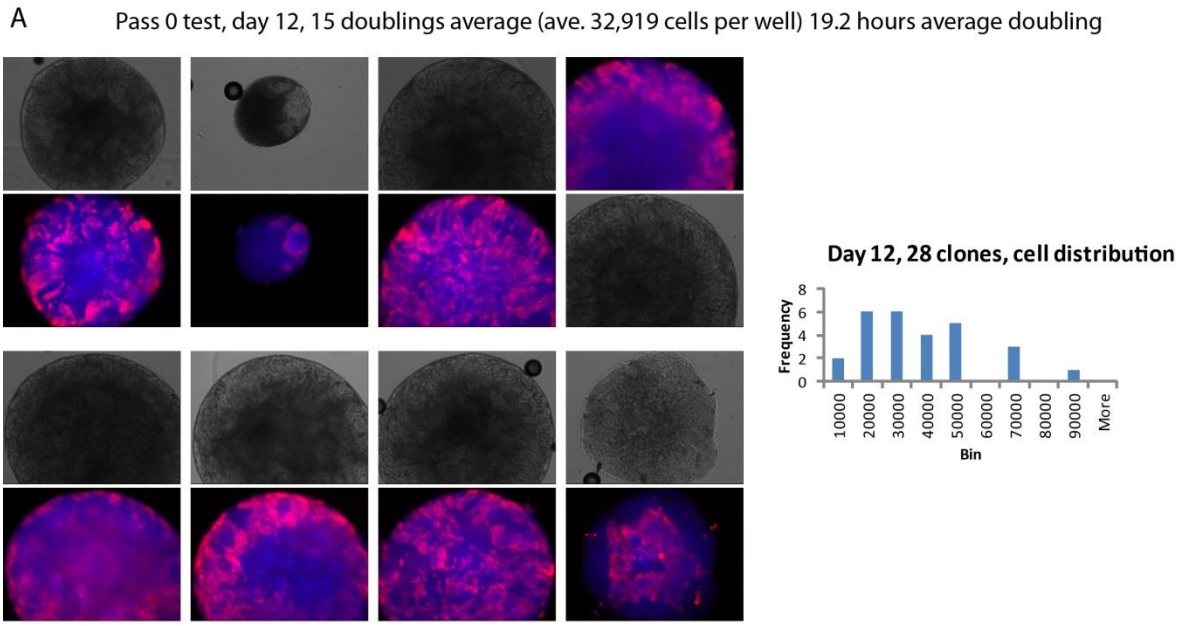


Figure S4| Expected and observed Poisson distribution of limiting dilution assay. A random sampling of 192 wells was chosen for the analysis and screened by light microscopy.

Figure S5



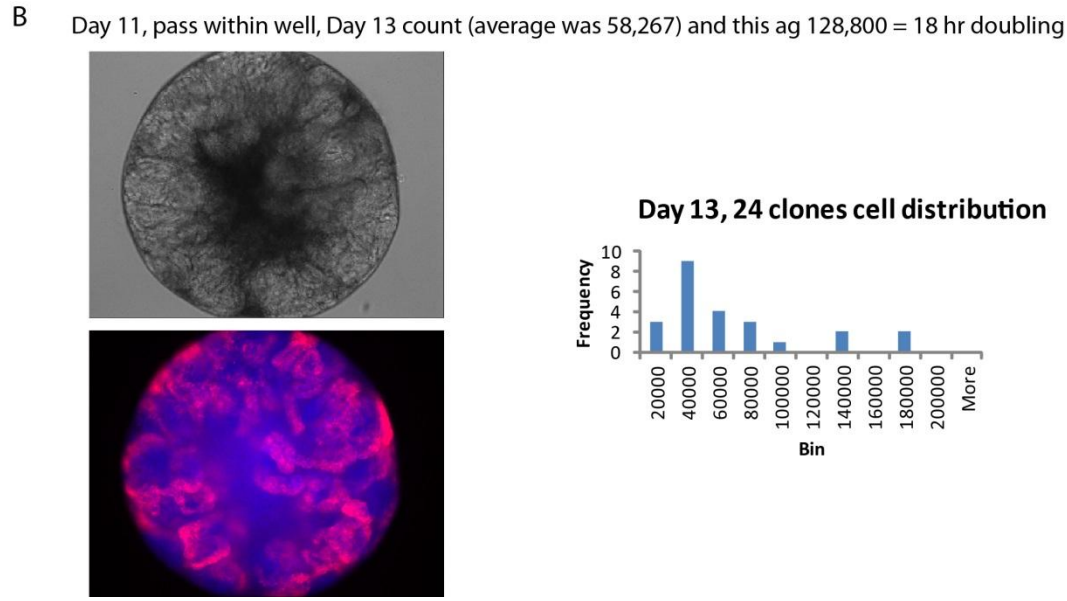


Figure S5| Test of the ability of clones expanded from a single cell to undergo tubulogenesis before and after passage.

(A) Single cells derived from a bulk population of CITED1 progenitors were seeded and grown in NPEM for 12 days. Colonies were dissociated, passed directly to 3D aggregate culture and treated with CHIR for 7 days. Aggregates were imaged by light microscopy, fixed and stained with lotus lectin (red) and DAPI (blue). Aggregates derived from 8 clones expanded in monolayer shown. Cell count distribution of 28 single cell derived colonies on day 12 shown in the graph to the right.

(B) Single cell derived colony dissociated on day 11, counted and cultured in monolayer for 2 more days before being passaged to 3D aggregate culture and treated with CHIR for 7 days. Aggregate imaged by light microscopy and fixed and stained with lotus lectin (red) and DAPI (blue). Cell count distribution of 24 single cell derived colonies on day 13 shown in the graph to the right.

Figure S6

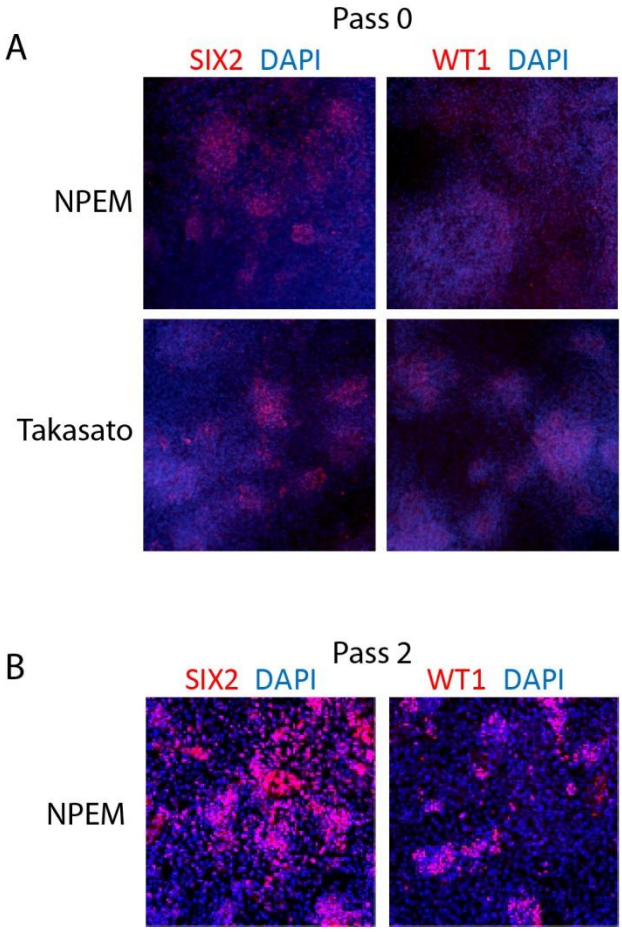


Figure S6| SIX2 and WT1 expression in human nephron progenitors derived from human ES cells after pass 0 and 2.
(A) Nephron progenitor cells immunostained for SIX2 and WT1 were differentiated for 5 days from H9 hESCs using the conditions reported by Takasato et al and switched to NPEM for an additional 5 days (top panels). Cells differentiated using the Takasato procedure for 10 days (bottom panels).
(B) Cells differentiated using the Takasato procedure were switched to NPEM after 5 days and expanded for 2 passages (1:8 split) with retained expression of WT1 and SIX2.

Figure S7

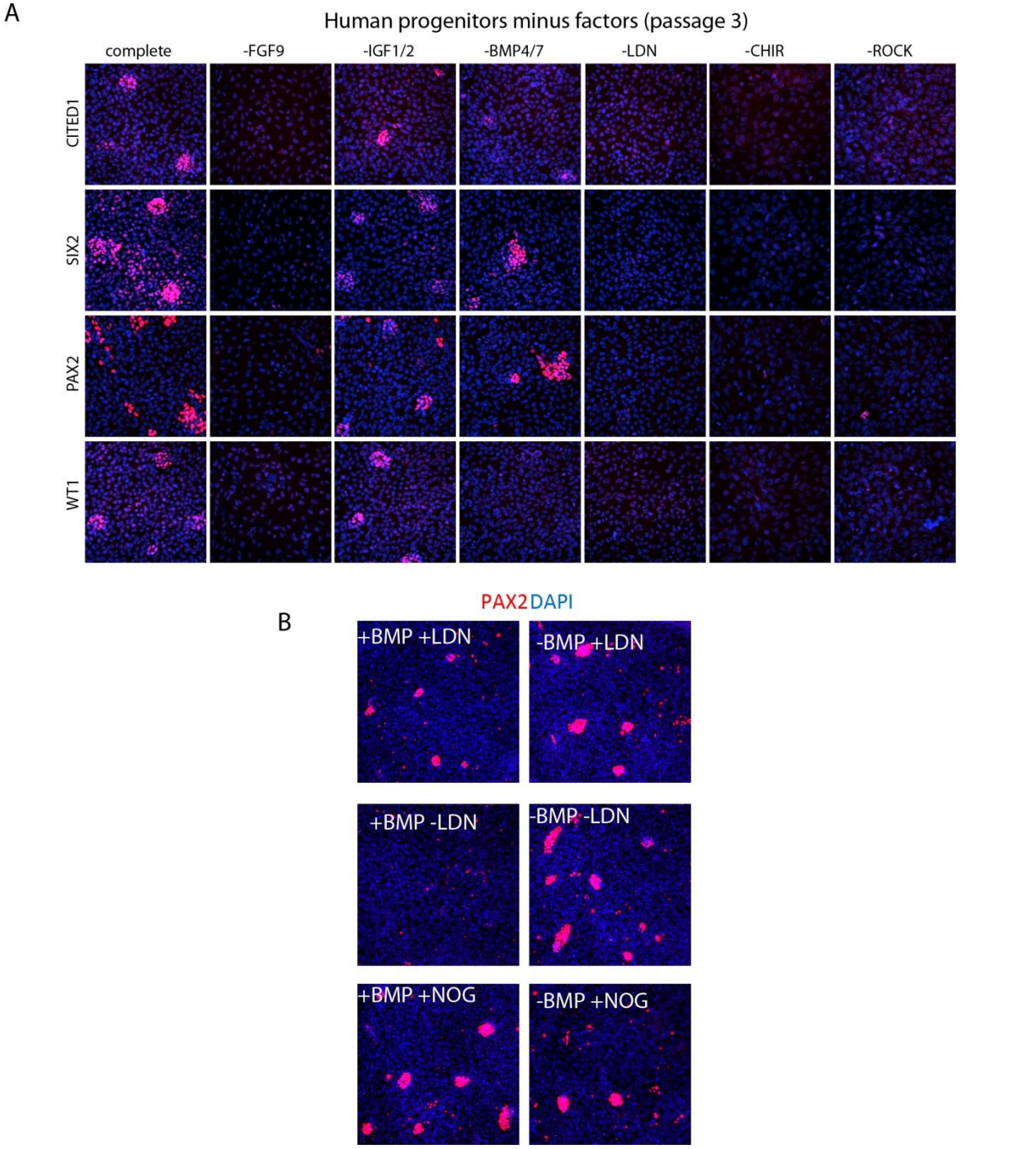


Figure S7| Nephron progenitor marker expression in human ES cell derived cultures grown in the absence of individual factors.
(A) Passage 3 progenitors were grown in NPEM in the absence of individual factors for 3 days in monolayer culture and immunostained for CITED1, SIX2, PAX2 and WT1. Antibody staining is shown in red and nuclear counterstaining with DAPI is shown in blue.
(B) Passage 3 progenitors were grown in NPEM in the absence of the indicated factors for 3 days in monolayer culture and immunostained for PAX2.